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THE
BIOLOGICAL BULLETIN

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THE MARINE BIOLOGICAL LABORATORY

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I. TRUSTEES

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II. ACT OF INCORPORATION

No. 3170

Commonwealth of Massachusetts

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, Henry B. Pierce, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised August 12, 1966)

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and nine Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-six Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed.

(B) Trustees *ex officio*, who shall be the Chairman, the Director, the Treasurer, and the Clerk.

(C) Trustees *Emeriti*, who shall be elected from present or former Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeriti* shall have all the rights of the Trustees, except that *Trustees Emeriti* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them except those chosen by the members, at any time. They may fill vacancies occurring in any manner in their own number or in any of the officers. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Broad of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These bylaws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the bylaws will be acted upon.

RESOLUTIONS ADOPTED AT TRUSTEES' MEETINGS

EXECUTIVE COMMITTEE

I. RESOLVED:

(A) The Executive Committee is hereby designated to consist of not more than ten members including the *ex officio* members who shall be the Chairman of the Board of Trustees, President, Director and Treasurer; six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term. (August 11, 1967)

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice President. A majority of the members of the Executive Committee shall constitute a quorum and a majority of those present at any properly held meeting shall determine its action. It shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine. (August 12, 1966)

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by the Board or law. (August 16, 1963)

(D) The Executive Committee shall keep appropriate minutes of its meetings, and its action shall be reported to the Board of Trustees. (August 16, 1963)

II. RESOLVED:

The elected members of the Executive Committee shall be constituted as a standing "Committee for the Nomination of Officers," responsible for making nominations at the Annual Meeting of the Corporation and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk).

IV. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory
Gentlemen:

1. The Business of Scientists is Science

The business of the Marine Biological Laboratory is to serve scientists. To be sure, scientists are people with individual social responsibilities and preoccupations. In like fashion, the MBL is a unit of a community with community interests and obligations.

The MBL was founded as a place for scholars, old and young, to pursue scientific inquiry by experiment, by observation, by cogitation and by communication. It is not an operation speaking with a single voice; it has no institutionally controlled scientific programs and its management is one for service rather than for casting plans for the molding of its future.

These thoughts add up to describe a remarkable institution which unfortunately defies shorthand description in this modern organized society. This is especially true where one might wish to speak of the results of the scientific work and education. MBL facilities are used by some 500 good scientists and to paraphrase the Constitution, all good scientists are equal. It matters not that, to quote another authority, some are more equal than others. The best that can be done, in fairness and justice, is to state that last season, the 81st. Session, scientific activity was marked by accomplishment and innovation. There were highlights—but as parts of a bright picture. It was a good year.

2. Our Building Program

The MBL building program exhibits some peculiarly biological properties: These are: (1) heterogenic growth, (2) specificity of biological time and (3) territoriality (perhaps even the territorial imperative.)

The first attribute refers to the difficulty in keeping buildings, food, scientists and laboratories in phase. I regret that difficulties in regulating the overall growth pattern are all too apparent. The second property is attested by our failure to have the new training building ready on schedule. Committees, architects, building trades and supplies all clearly have different time constants and it is well nigh impossible to predict in advance specific growth rates. We offer our apologies for the inconvenience of the delay and extend our appreciation to the course heads for the resourceful way in which they responded.

The new dining-dormitory complex is delayed another year. I hope we can make that schedule. The causes of the delay, which clearly relate to territoriality, need not be reviewed again. However, the discussions relating to our plan to place the new living amenities on the Breakwater site were sufficiently vigorous to enlist the attention of a number of friends, especially in the Penzance Point area. With their support and encouragement and the wise and masterly efforts of Mr. Swope and Mr. Smith, funds were raised and land was purchased to enable MBL to place the dining-dormitory complex on the shores of Eel Pond. This site has notable logistic advantages over the Breakwater site, especially during periods of inclement weather, and the newly planned structure will serve our summer population well and be a boon to all the increasing number of fall, winter and spring users. The MBL owes a great debt of gratitude to those whose efforts made this plan possible. I especially wish to pay tribute to the fine cooperation of those who agreed to move their homes in order to allow MBL to develop its new plans. The new plan accomplishes the purposes so wisely set forth by Dr. Armstrong, Dr. Parpart and others, and adds the great advantage of immediate proximity to the rest of our operations. Plans will be available for inspection in the summer of 1969.

3. *MBL Policy and the Future*

The Marine Biological Laboratory exists to provide service to scientists to solve fundamental problems in life sciences and to give training to developing scientists. Due to its location and to the reasons for selecting the site, the MBL has always depended heavily on life in the oceans but has never been limited to the exclusive use of marine forms.

In the last twenty years or so, the importance of oceanic life forms as part of the marine environment has been favored with a great deal of public attention relating especially to such matters as pollution, productivity, fishing, extraction of chemicals and the like. Dramatic attention has been called to all these aspects most recently by the report of the Commission on Marine Science and Technology. What is the role of MBL, if any, to respond to the proposed program of the Commission? All of us as citizens, scientists and Corporate members of MBL need to do some heavy thinking.

At the outset, the MBL was founded as a user-supported operation. Major costs were met from the Laboratory fees contributed by investigators; expansive steps were accomplished by securing major bits of financial support for the specific items such as the Crane building, the Library addition and so forth. What are our plans for the future? What will be our funding pattern?

Over the years this pattern has changed quantitatively. User fees account for less than a third of the costs, endowment has risen somewhat but the major expansion in operating funds has come from the grants-and-contracts system, primarily from Federal sources. This increasing dependence on public funds, especially in the present political climate will demand and, indeed, is now demanding that the MBL make clearly understandable and visible its role in the rapidly expanding scientific effort, especially as related to marine sciences. Thus, it is imperative that we keep our objectives clearly in mind and that we stand ready to interpret them in terms of the development of science as a whole. We must be able to explain to a changing social scene why we exist and are worthy of public support.

Especially I urge attention to the extreme importance of keeping our overall management a matter of concern of all user-scientists and Corporation members. As the fraction of support contributed by users declines, there is an inevitable decline in the degree of user-responsibility. The phase "why don't *they* do something about" recurs with distressing frequency, "they" referring to the visible management. The MBL can survive in its present form only if responsibility for the Institution is shared by all. Otherwise there will be an inevitable tendency to drift into the usual programmatic, managerial-oriented laboratory so characteristic of big science today.

4. *The MBL and Community Responsibility*

Some seventy years ago, Woods Hole was a thriving fishing and fertilizer community served by an active railroad, a large fleet of small boats, several assorted small businesses and including a few tourists and a few scientists. Over the years, commercial fishing has practically disappeared; fertilizer is imported, not manufactured; the railroad has gone; tourists have become a major seasonal factor; small business has virtually disappeared; and the scientific establishment has become a dominant economic and political factor.

All of us regret the passing of Woods Hole as a charming seaside fishing village and, therefore, share the responsibility for its future development as a major scientific center. It would be a grievous error to assume that Woods Hole, the community, must become only a collection of institutions overrun periodically with transient visitors. Rather, it behooves the MBL and its sister institutions to work with the other segments of the community, and even more important, it should be the responsibility of the members of the scientific community to increasingly play a role as informed and concerned citizens of Woods Hole.

Last year the MBL became involved in community matters in a rather distressing fashion. Our wholly admirable plans for erecting a dining-dormitory complex on the Breakwater site suddenly became a symbol of the oppression of the individual citizen by an Institution. Happily, community awareness won on all sides with the result of the new building program already outlined.

It is quite apparent that MBL has another community problem on its hands—the beach. Stony Beach (including parking space and tennis courts) is wholly owned by MBL. In part, this land constitutes a legal trust for MBL to keep the beach open to Woods Hole users, including scientists; the moral trust is imperative.

Those of you who are curious about the history of beach rights in Woods Hole are urged to read the Collecting Net starting about 1930. At that time, there was danger that all of the beach would be fenced off except for a narrow stretch with a right-of-way set up in trust by Mrs. Fay. A Committee, apparently of the Woods Hole Protective Association, but including many MBL founding fathers, went to work, and as a result of much discussion coupled with the wise generosity of such individuals as Drs. Meigs, Strong and Clowes, titles to the present property were turned over to MBL, but with the strong implication of the moral obligation to maintain the beach of the commonweal. Sadly, there appears to be an appreciable number of individuals, residing nearby, who consider the beach a nuisance. As of now, MBL policy is to cooperate with town officials to the fullest extent to ensure an orderly public beach. The community problem emerging is one in which all members of the community—users, owners, neighbors—must share responsibility. The MBL institutional posture must be that the beach, along with such other amenities as the sundial park must be kept open for public use. Woods Hole, its beaches and its parks, must not be allowed to become the exclusive domain of a few.

5. *Fund Drive*

During the past year, because of the need of concentrating on the special funds needed for procuring and developing the Eel Pond site, the general fund drive has gone into slow motion: $137.000—having been pledged; $66,000— having been raised; amounting to a total of $203,000; and we now intend to renew our efforts. All Corporation Members are cordially invited to contribute and to proselytize any sympathetic friends you might have.

6. *Personnel*

The MBL has a plant valued at $4,246,441. The current building program amounts to about $7,000,000. We maintain 75 buildings counting cottages and other residences. Our staff manages all this and also takes care of supplying materials and equipment, feeding, housing and otherwise tending some 500 individualistic scientists and their dependents. Our staff, headed by our General Manager, is superb and we all owe our staff members a debt of gratitude. Their virtuosity is equalled only to their equanimity, poise and good humor.

The Laboratory bears a deep sense of gratitude to Dr. Donald P. Costello and Dr. Catherine Henley for service to THE BIOLOGICAL BULLETIN. We are fortunate in that Dr. W. D. Russell-Hunter was able to accept the Editorship of the BULLETIN. We are also indebted to Dr. Russell-Hunter for the years of service he has rendered to the Laboratory as Head of the Invertebrate Zoology Course. We look forward to the further innovations that will be introduced into the course beginning in 1969 under the leadership of Dr. James Case. Dr. William Hillman presented a stimulating program in the Marine Botany course during the interim year of 1968 when he assumed responsibility for that course. Dr. Harold W. Siegelman is taking over the Botany course in 1969 and, likewise, we anticipate the future development of this course under Dr. Siegelman.

1. MEMORIALS

FERDINAND J. M. SICHEL

By Rudolf T. Kempton

With the death of Dr. Ferdinand J. M. Sichel on April eleventh of this year, the Laboratory lost a devoted supporter who first worked here in the summer of 1927 and became a member of the Corporation thirty-five years ago. In his earlier years here he was very active in the MBL Club, and he served on the housing, library, apparatus and other committees. His was an interest that never flagged, and in those years in which he was not able to spend the summer at Woods Hole he always made at least a visit to the Laboratory, often at the time of the Corporation meetings, of which he missed only a few.

Dr. Sichel was born in Hamilton, Ontario, in 1906; he became a United States citizen in 1940. His undergraduate study was at McGill University. From 1928–1934 he carried on graduate study in the Biology Department at Washington Square College, New York University, where he took his master's degree and doctorate with Dr. Robert Chambers. His research for the doctorate dealt with the micromanipulation of single skeletal muscle fibers. He never lost his interest in excitable tissues, especially muscle.

By the time he took his doctorate in 1934, he already had had considerable teaching experience, enough to have developed a great interest in the education of young men and women. He had been a Démonstrator in Botany at McGill for one year, and an assistant instructor at New York University for five years. He added to this successively a year as instructor in the Zoology Department at the University of Pennsylvania; a year at the Johnson Foundation for medical physics as a Postdoctoral Fellow of the Royal Society of Canada; and a year of teaching physiology at the Howard University School of Medicine. In addition, from 1933–1944 he taught micromanipulation each summer in the MBL physiology course. Thus when he went to the University of Vermont School of Medicine in 1937 he was a teacher with experience beyond his years. He was destined to serve at that institution throughout the remainder of his life. Starting as instructor, he rose to a full professorship in 1944 when he was made chairman of the Department of Physiology. He was a member of many societies, biological, physical and medical, and was a thirty-second degree Mason.

Dr. Sichel had strong convictions concerning the necessity for medical students to receive rigorous basic training, to develop a feeling for the scientific approach, and to achieve a firm physiological foundation. Being a perfectionist, he took endless pains in the presentation of physiological material to the students. His lectures were known as perhaps the best given at the institution, meticulous in their logic, clearly with a step-by-step development, presented with the greatest possible clarity. Added to this was his deep interest in students as a group and as individuals. The combination produced a very high level of student respect and appreciation. Personally I have encountered a number of graduates from the medical school, and they were unanimous in their regard for "old Doc Sichel."

As Chairman of the Physiology Department he was responsible for the establishment and development of a program for the Master's degree, and subsequently a Ph.D. program. More recently he developed a program in Biophysics associated with the

Physiology Department. At the time of his death he had given up the chairmanship of the whole department and had become chairman of the Division of Biophysics in the Department of Physiology and Biophysics.

As a stimulator of research Dr. Sichel's influence was wider than his department. One example is seen in the statement of the Vermont Heart Association upon their establishment of the "Ferdinand J. M. Sichel Fellowship in Cardiovascular Disease. . . ." They said ". . . he was instrumental in establishing a vital heart cornerstone, the formulation of Vermont Heart Research Policies . . . a diligent and conscientious member of our Research Committee for more than seven years, and its chairman for three years. . . . His pensive, alert mind stimulated purposeful discussion and debate, marking milestone after milestone. . . . Very aptly our research fellowship is being designated in his honor."

He was a man of many facets, and one was his hobbies. He was an ardent and accomplished skier, a sport he was forced to discontinue in 1947 after suffering a coronary attack. He did continue dry-fly fishing, in which he was highly skillful. Some of his hobbies were less taxing physically. He found great relaxation in mathematical puzzles; he enjoyed working out electron circuitry for various purposes; and he would travel many miles for the opportunity to listen to good Scottish pipers. Above all, he loved good conversations with small groups of friends.

For those of us who know him for many years our memories are clear. His interest in people, his quiet voice, his very real but subdued sense of humor, his wide scientific interests, will remain with us for the rest of our lives. We believe we can understand something of the sense of loss being experienced by his widow Elsa Keil Sichel, and his daughter Enid, a graduate student in physics.

2. The Staff

EMBRYOLOGY

I. Instructors

Malcolm S. Steinberg, Professor of Biology, Princeton University, in charge of course

John M. Arnold, Assistant Professor of Cytology, Pacific Biomedical Research Center, University of Hawaii

Max Burger, Assistant Professor of Biology, Princeton University

Ralph Hinegardner, Associate Professor of Biology, University of California at Santa Cruz

Irwin R. Konigsberg, Professor of Biology, University of Virginia

James W. Lash, Associate Professor of Anatomy, University of Pennsylvania

Hans Laufer, Associate Professor of Zoology, University of Connecticut

Raymond Rappaport, Professor of Biology, Union College

II. Consultant

Everett Anderson, Professor of Biology, University of Massachusetts

III. Laboratory Assistants

Burr G. Atkinson, Jr., University of Connecticut

Norman A. Granholm, University of Oregon

IV. Lectures

M. S. Steinberg	Introduction to the course
I. R. Konigsberg	Teleosts I
	Teleosts II
John Wourms	The annual fish egg: a dispersion-reaggregation system subject to multiphasic diapause
Gertrude Hinsch	Fertilization
Everett Anderson	The fine structure of eggs
Antone Jacobson	Control of heart determination by tissue interactions and by tissue extracts
Hans Laufer	Crustaceans I
	Crustaceans II
	Crustaceans III
R. Hinegardner	Echinoderms I
	Echinoderms II
Paul Gross	Control of macromolecule synthesis during early development in echinoderms
Lionel Rebhun	The mitotic apparatus
Lewis Wolpert	Cellular basis of sea urchin morphogenesis
Raymond Rappaport	Cytokinesis I
	Cytokinesis II
M. Burger	Biosynthesis of structural elements of cell walls and membranes
M. S. Steinberg	Self-assembly of multicellular complexes
Vera King Farris	Molluscan cells: dissociation and reaggregation
J. Arnold	Spiralians I
	Spiralians II
	Spiralians III
	Spiralians IV
M. S. Steinberg	Morphogenetic phenomena in sponges
	Coelenterates I
	Coelenterates II
Lewis Wolpert	Cellular basis of sea urchin morphogenesis
J. Lash	Ascidians I
	Ascidians II
Kirby Smith	RNA and protein synthesis during early ascidian development
J. Lash	Ascidians III
Lionel F. Jaffe	The early development of *Fucus*: prototype of localization
Bernice Grafstein	Transport of materials in nerve fibers
Roger Milkman	Genetics and development of *Botryllus schlosseri* (Ascidiacea)
Paul B. Weisz	The significance of larvae
Jack Collier	Biochemistry of the Ilyanassa embryo
Igor Dawid	The DNA components of amphibian oocytes
L. Dennis Smith	Protein synthesis in early cell division of amphibians
Gerald Weissman	Lysosomes and template changes in transforming lymphocytes
Earl Frieden	Biochemical approaches to the problem of amphibian metamorphosis
Maurice Sussman	The programmed accumulation and disappearance of enzyme activities

PHYSIOLOGY

I. Consultants

Merkel K. Jacobs, Professor of Physiology, University of Pennsylvania
Albert Szent-Györgyi, Director, The Institute for Muscle Research, Marine Biological Laboratory
W. D. McElroy, Director, McCollum-Pratt Institute, The Johns Hopkins University
J. Woodland Hastings, Professor of Biology, Harvard University

II. Instructors

Andrew G. Szent-Györgyi, Professor of Biology, Brandeis University, in charge of course
Sydney Brenner, Medical Research Council, Laboratory of Molecular Biology, Cambridge, England
Roderick K. Clayton, Professor of Biophysics, Cornell University
Seymour S. Cohen, Professor of Biochemistry, University of Pennsylvania
Hugh E. Huxley, Medical Research Council, Laboratory of Molecular Biology, Cambridge, England
Maurice Sussman, Professor of Biology, Brandeis University
David A. Yphantis, Professor of Chemistry, University of Connecticut

III. Special Lecturers

Harlyn Halvorson, Professor of Bacteriology, University of Wisconsin
Shinya Inoué, Professor of Biology, University of Pennsylvania
Hans Kornberg, Professor of Biochemistry, University of Leicester, England
John G. Nicholls, Associate Professor of Physiology, Yale University Medical School
K. E. Van Holde, Professor of Physical Chemistry, University of Oregon

IV. Staff Associates

Annemarie Weber, Department of Biochemistry, St. Louis University
Raymond E. Stephens, Department of Biology, Brandeis University
James R. Bolton, Department of Chemistry, University of Minnesota
Celia E. Freda, Department of Therapeutic Research, University of Pennsylvania
Raquel Sussman, Department of Biology, Brandeis University
Robley C. Williams, Jr., Department of Biology, University of Connecticut
Michael K. Reedy, School of Medicine, University of California at Los Angeles

V. Research Assistants

B. J. Clayton, Department of Genetics Development and Physiology, Cornell University
Richard Kessin, Department of Biology, Brandeis University
Robert M. Levy, Washington University Medical School
Anne McConville, Department of Therapeutic Research, University of Pennsylvania
Ellen Rayner, Department of Biology, Brandeis University

VI. Course Assistants

John I. Clark, Department of Biological Structure, University of Washington
Margaret Ketchum, College of Liberal Arts, Boston University

VII. Lectures

Andrew G. Szent-Györgyi	Muscle contraction I
	Muscle contraction II
Bernard R. Baker	Active site directed inhibition of dehydrofolate reductase
Annemarie Weber	Control of contraction
R. K. Clayton	An outline of the photosynthetic mechanism
	Physical processes in photosynthesis
George Klein	Brief survey of tumor immunology
J. M. Olson	Evolution of photosynthetic electron transfer
Seymour S. Cohen	Origin and properties of some phage-induced enzymes
	Essential and inessential early functions in t-even phage infection
	Control of gene expression in t-even phage infection
John R. Platt	First stage electrical effects in vision
G. Cantoni	Structure and function of t-RNA
Maurice Sussman	Slime molds XLVI
	Slime molds XLVII
	Slime molds XLIX
Boris Rotman	Skewed distribution of galactosidase among uninduced cells of *E. coli* and its possible relation to *in vivo* activity of m-RNA
Matthew R. Messelson	Replication of DNA
Harlyn O. Halvorson	Temporal control of enzyme synthesis in microorganisms
Shinya Inoué	Mitotic spindle
R. E. Stephens	Microtubules
Hugh E. Huxley	The structure of the filaments in striated muscle: X-ray and electron microscope evidence I
	The structure of the filaments in striated muscle: X-ray and electron microscope evidence II
Michael K. Reedy	Arrangement and behavior of crossbridges in insect flight muscles
Sydney Brenner	Gene regulation I
	Gene regulation II
	Gene regulation III
Alfred Tissiere	Ribosomal proteins
John G. Nicholls	Electrical signalling in the nervous system
	Neural organization in the visual system
	Long-term changes in synaptic transmission
David A. Yphantis	Physical approaches in biochemistry I
	Physical approaches in biochemistry II
	Physical approaches in biochemistry III
Albert Szent-Györgyi	Bioelectronics: regulations, defense and cancer
K. E. Van Holde	Polynucleotide structure I. Circular dichroism studies of synthetic polynucleotides
	Polynucleotide structure II. Sedimentation studies of monomer interactions
Howard Holtzer	Myosin synthesis, fusion and the mitotic cell
J. W. Hastings	The chemistry and biology of excited singlet oxygen
S. Roseman	Relationship between a bacterial phosphotranspherase system and sugar transport
E. Kennedy	Biochemical study of lactose transport in *E. coli*

Jack Strominger	Role of the membrane in bacterial cell wall synthesis and its interaction with antibiotics
Henry R. Mahler	Structural proteins in membranes
L. Warren	Composition and metabolism of animal cell surface membranes
W. Loewenstein	Membrane functions: intercellular communication
E. Kravitz	Study of synaptic chemistry in single neurons
R. Levine	Mode of action of insulin
S. Luria	Colicines and the bacterial envelope
Paul Gallop	Maturation of connective tissue
Yngve Zotterman	Recording of the electrical response from human taste nerves: the relation between neural and perceptual intensity
George Wald	Molecular basis of visual excitation
J. Aschoff	Rhythms in humans
Hans Kornberg	Control of bacterial carbohydrate I
	Control of bacterial carbohydrate II
B. Pullman	Aspects of the electronic structure of purine and pyrimidine bases and of their interaction
B. D. Davis	The role of a ribosome dissociation factor in the polysome cycle
J. Gross	Morphogenesis and collagen structure

VIII. Special Seminars

I. M. Klotz	Subunit structure and subunit interaction in proteins: Hemerythin
M. Moody	The structure and contraction of the tail of *t*-even phages
A. Oplatka	The application of irreversible thermodynamics to muscle contraction
David White	Mechanical studies of insect flight muscles
Robert M. Bock	Control of translation
Walter Hill	X-ray scattering and sedimentation: equilibrium studies of *E. coli* ribosomes
Margaret Miovic	Protein initiation and T-2 infection
S. Ebashi	Excitation contraction coupling I
M. Endo	Excitation contraction coupling II

EXPERIMENTAL BOTANY

I. Instructors

William S. Hillman, Brookhaven National Laboratory, in charge of course
Robert R. L. Guillard, Woods Hole Oceanographic Institution
John M. Olson, Brookhaven National Laboratory
Harold W. Siegelman, Brookhaven National Laboratory
Robert T. Wilce, University of Massachusetts, Amherst

II. Special Lecturers

G. B. Bouck, Yale University
P. B. Green, University of Pennsylvania
L. Provasoli, Haskins Laboratories, New York

M. Gibbs, Brandeis University
J. E. Leggett, U. S. Department of Agriculture
F. A. Loewus, State University of New York at Buffalo
N. Lazaroff, State University of New York at Binghamton

III. Laboratory Assistant

Robert E. Lee, University of Massachusetts

IV. Lectures

R. T. Wilce	Chlorophyta
	Phaeophyta
	Rhodophyta
	Cyanophyta
R. R. T. Guillard	Influence of environment factors on phytoplankton
	Distribution of light, of plants and of plant pigments in the sea
	The influences of light regime, temperature, salinity and of organic and inorganic nutrition on photosynthesis and other physiological responses on marine algae
J. M. Olson	Photosynthesis
	Relationships between photosynthetic bacteria and blue-green algae
	Pigment systems
	Electron transport systems for generation of ATP
W. S. Hillman	Light quality in photomorphogenesis and photoperiodism: phytochrome
	Phytochrome: *in vivo* assays and physiological interpretations
	Endogenous circadian rhythms and the timing mechanism in photoperiodism
	Speculations on evolution and comparative biology of photomorphogenesis and photoperiodism in algae, in higher plants, and in animals
H. W. Siegelman	Modern isolation and separation techniques for small and large molecules
	Gel filtration, centrifugation, thin-layer chromatography and electrophoretic methods
	Isolation, purification and properties of algal chromoproteins I
	Isolation, purification and properties of algal chromoproteins II
	Isolation, purification and properties of the algal bile pigments
	In vivo, properties, isolation, purification and properties of protochlorophyll holochrome

INVERTEBRATE ZOOLOGY

I. Consultants

Frank A. Brown, Jr., Morrison Professor of Biology, Northwestern University
Libbie H. Hyman, The American Museum of National History

Clark P. Read, Professor of Biology, Rice University
Alfred C. Redfield, Woods Hole Oceanographic Institution

II. Instructors

W. D. Russell-Hunter, Professor of Zoology, Syracuse University, in charge, of course
George G. Holz, Jr., Professor of Microbiology, State University of New York, Upstate Medical Center
Norman Millott, Professor of Zoology, Bedford College, University of London, England
Frank M. Fisher, Jr., Associate Professor of Biology, Rice University
Robert K. Josephson, Associate Professor of Biology, Case Western Reserve University
Jonathan P. Green, Assistant Professor, Division of Biological and Medical Sciences, Brown University
Meredith L. Jones, Curator-in-charge, Division of Worms, United States National Museum, Smithsonian Institute
Irwin W. Sherman, Associate Professor of Zoology, Department of Life Sciences, University of California, Riverside

III. Laboratory Assistants

Darrell R. Stokes, University of Hawaii
Robert J. Avolizi, Syracuse University

IV. Lectures

W. D. Russell-Hunter	Orientation, and functional homologies in invertebrates
Darrell R. Stokes and Robert J. Avolizi	Laboratory orientation
George G. Holz, Jr.	The nature of the Protozoa
	Flagellates I
W. D. Russell-Hunter	Littoral ecology: Theoretical and physiological
George G. Holz, Jr.	Littoral ecology: Practical and pragmatic
	Flagellates II—Sarcodines I
	Sarcodines II
	Ciliates
Irwin Sherman	Porifera
Robert K. Josephson	Cnidaria I—Introduction to the Cnidaria and Ctenophora
	Cnidaria II—Nematocyst structure and function feeding
	Nervous systems and behavior in jelly-fish and sea anemones
	Seminar—nerve nets and conducting epithelia in Hydrozoa
Frank M. Fisher, Jr.	Turbellaria and Trematoda
	Cestoda and Rhynchocoela
W. D. Russell-Hunter	Mollusca I—General molluscan organization—functioning of mantle-cavity in Gastropoda
	Mollusca II—Gastropoda (continued): Mantle-cavity and feeding mechanisms in Bivalvia
	Seminar—Some problems of mechanics in molluscs
	Mollusca III—Adaptations in bivalves: Aspects of general physiology of gastropods and bivalves
Frank M. Fisher, Jr.	What about rhynchocoels?

W. D. Russell-Hunter	Mollusca IV—Functional morphology in Cephalopoda and minor groups
	Mollusca V—Functional morphology in Amphineura, *Neopilina*, archetypes and ancestors
Frank M. Fisher, Jr.	Aschelminthes
	Entoprocta, Ectoprocta
Meredith L. Jones	Annelida: Introduction, morphology, classification
W. D. Russell-Hunter	Seminar—Time and space in fresh waters: molluscan evolution
Meredith L. Jones	Annelida: Feeding, circulation, osmoregulation
	Annelida: Nervous system, reproduction
	Arthropoda I
Jonathan P. Green	Arthropoda II
	Arthropoda III
Meredith L. Jones	On *Caobangia*, an aberrant fresh-water sabellid polychaete from southeast Asia
Jonathan P. Green	Arthropoda IV
Norman Millott	Echinodermata I—Radiate forms (asteroids and ophiuroids)
	Echinodermata II—Globular forms (echinoids)—the functions of spines and podia
	Echinodermata III—Globular forms II (holothurians) stalked forms (crinoids)
	Echinodermata IV—Development and phylogeny
Irwin Sherman	Protochordata I—Introduction, feeding, digestion neuromuscular system
A. Farmanfarmaian	Intestinal absorption in *Thyone*
Norman Millott	Dermal light sense
Irwin Sherman	Protochordata II—Circulation, respiration, excretion and reproduction
W. D. Russell-Hunter	One approach to the zoöplankton
Robert Robertson	Pyramidellids—ectoparasitic gastropods at Woods Hole

MARINE ECOLOGY

I. Consultants

Melbourne R. Carriker, Marine Biological Laboratory
Edwin T. Moul, Rutgers University
John H. Ryther, Woods Hole Oceanographic Institution

II. Instructors

Lawrence B. Slobodkin, Department of Zoology, University of Michigan, in charge of course

Edward R. Baylor, Woods Hole Oceanographic Institution

Dennis J. Crisp, Director, Marine Science Laboratories, University College of North Wales, U. K.

Howard L. Sanders, Woods Hole Oceanographic Institution

W. Rowland Taylor, Department of Oceanography and the Chesapeake Bay Institute, The Johns Hopkins University

Gotram Uhlig, Biologische Anstalt Helgoland, Hamburg, Germany

III. Special Lecturers

Luigi Provasoli, Haskins Laboratories, New York
David C. Grant, Systematics-Ecology Program, Marine Biological Laboratory

IV. Laboratory Assistants

John F. Boyer, Department of Zoology, University of Chicago
Herman F. Bosch, Department of Oceanography, The Johns Hopkins University

V. Lectures

L. B. Slobodkin	Introduction
W. Rowland Taylor	The marine environment
	Phytoplankton I
	Productivity
	Phytoplankton II
	Phytoplankton III
E. R. Baylor	Formation of organic particles
	In situ abundance of zooplankton
	Zooplankters' response to light and chemicals
	Sound in the ocean
Ernest Mayr	Species diversity
Kevin Little	Searching behavior of *Artemia*
L. B. Slobodkin	How to succeed at evolution
David C. Grant	The ecology of Barnstable Harbor
L. B. Slobodkin	Population growth
	Ecology and people
L. Provasoli	Axenic culture of Crustacea
Howard L. Sanders	Animals, sediments and feeding types
D. C. Rhodes	Sediment-water interface and biological activity
Howard L. Sanders	Salinity, hydrography and distribution of estuarine animals
Roger H. Green	The structure of an intertidal community
Howard L. Sanders	The biology of the deep-sea benthos
	Benthic diversity—A comparative study
Gotram Uhlig	Introduction to the benthic microfauna
	Feeding types and food chains in the benthic microfauna
	Ecological relations of life cycles in the benthic microfauna
	Methods in studying interstitial microfauna
L. B. Slobodkin	Predation and energetics
Gotram Uhlig	Studies of the folliculinids (Ciliata)
	Ecology and life cycle of *Noctiluca miliaris*
E. O. Wilson	Experimental Biogeography
D. J. Crisp	Classification and significance of larvae
	Plankton shapes, behavior and nutrition
	Behavior of larvae at settlement
	Gregarious and territorial settlement
	Substrate selection by larvae of deposit—living species
	Chemical inducements to settlement and metamorphosis

SYSTEMATICS–ECOLOGY PROGRAM

The Staff

Director: Melbourne R. Carriker
Resident Systematist: Robert P. Higgins
Resident Ecologists: Roger H. Green and David K. Young
Assistant Ecologist: Katharine D. Hobson
Resident Biologist: David C. Grant
Postdoctoral Fellows and Research Associates: David G. Cook, Celia R. Haigh, Norman R. Sinclair and William C. Summers
Graduate Research Trainees: William R. Cobb, William D. Hummon, Michael L. May, Allan D. Michael, Leland W. Pollock and James R. Sears
Visiting Investigators in Residence: Edward L. Bousfield, Ralph O. Brinkhurst, Louise Bush, Howard H. Chauncey, Edwin T. Moul, Philip Person, Harold H. Plough, Ruth D. Turner, Robert T. Wilce and Donald J. Zinn
Consultants: William R. Taylor, Ruth D. Turner and Robert T. Wilce
Curator: Johanna M. Reinhart
Technical Field Assistants: Paul A. Shave, Peter J. Oldham
Artist: Ruth von Arx
Captain, R/V A. E. Verrill: James P. Ostergard
Mate, R/V A. E. Verrill: Peter Graham
Administrative Assistant: Hazel W. Fermino
Program Secretary: Eva S. Montiero
Research Assistants: Linda Gleye, Theodore J. Grant (bibliographer), David J. Hartzband, Maryln Hummel, Leilani McCoy, Richard R. McGrath, Charles R. McKay, Ellen P. McReynolds, Anne Richards, Armand G. Roberge, Mary Romano, Carol Q. Schwamb, Steven H. Slomka, Anne Smarsh, Michael A. Soukup, Dirk Van Zandt
Visitor: Mohamed Hyder

Seminars (winter included)

Ehrhard Voigt	Paleontological research at Hamburg University
Johanna M. Reinhart	Behavior of *Gastropteron pacificum*
Nelson Marshall	Trophic studies on shoal benthic environments
Charles L. Wheeler	The Woods Hole Aquarium: old and new
Madelene E. Pierce	The effects of several herbicides on plants and invertebrate animals in Nobska Pond
James E. Craddock	Comments on mesopelagic fishes
David G. Cook	Systematic studies on some oligochaeta
Robert E. Hillman	Mucus histochemistry of *Mercenaria mercenaria* mantle edge
Roland L. Wigley	Discussion of comparison between Van Veen and Smith-McIntyre grab samplers
Steven Obrebski	Parasite induced population fluctuations in *Transennella* and *Gemma*
Kenneth R. H. Read	Beneath New England waves
Sanford A. Moss	Recent research on physiological and behavioral interactions between fish schools and the environment
John M. Arnold	An analysis of the development of the squid *Loligo pealei*
William R. Cobb	The manner in which boring sponges (Clionidae) penetrate calcareous substrates

Edward B. Cutler	Systematics, ecology and distribution of the western North Atlantic sipunculids
David W. Frame	Seasonal variation of biota attached to test panels in the Cape Cod Canal
Sarah Haigler	Boring behavior of *Polydroa websteri*
Robert P. Higgins	Instant animal
Alfred W. Senft	Radioautograph studies on the uptake of carbon 14 proline in *Schistosoma mansoni*
Robert Edwards	The fisheries of the New England area
D. Craig Edwards	The ecology and behavior of some prosobranchs
Paul A. Shave	Some problems concerning tropical collecting
William D. Hummon	An assessment of the Hutchinsonian Niche concept (or, a bit of niche—pickin')
Johanna M. Reinhart	The Albatross seeks a snark
Kenneth J. Boss	Systematics of the Vesicomyidae (Mollusca, Bivalvia)
D. Gordon Mott	Dynamic population systems—model construction and analysis
Wesley N. Tiffney	*Tulostoma brumale* Persoon, a new resident of the Cape Ann Dunes
William C. Summers	Early growth and size distribution of *Loligo pealei*
William B. Nutting	Parasitic mites (Demodex) of mammals—oceans unknown
Jan Hahn	Productivity in the ocean and sediments on the continental shelf
Roger H. Green	Short tour of southern Africa
John H. Crowe	Some aspects of the water relations of a bryophilous tardigrade
Ralph O. Brinkhurst	The role of amino acid and bacteria in the nutrition of tubifid oligochaetes
James R. Sears	Reproduction of several benthic marine algae with special reference to phytogeographic distribution
Meredith L. Jones	On the reproduction and reproductive morphology, *inter alia*, of *Streblospio benedicti* Webster
David C. Grant	The biotic census of Cape Cod Bay
Robert P. Higgins	The kinorhynch genus *Cateria*: its adaptations and evolutionary significance
Ron Kenny	Some temperature relationships of intertidal species of *Acmaea*
David K. Young	Seston-sediment-invertebrate relationships in Buzzards Bay

THE LABORATORY STAFF

Homer P. Smith, general manager

Miss Jane Fessenden, Librarian
Carl O. Schweidenback, Manager, Supply Department
John J. Valois, Assistant Manager, Supply Department
Frank A. Wildes, Accounting Manager
Robert Kahler, Superintendent, Buildings and Grounds
Robert Gunning, Assistant Superintendent Buildings and Grounds
Robert B. Mills, Manager, Department of Research Service

GENERAL OFFICE

Edward J. Bender
Mrs. Vivien R. Brown
Mrs. Florence Butz
Mrs. Colleen P. Hall
Mrs. Ann Loomis
Mrs. Vivian I. Manson
Miss Elaine Perry
Mrs. Lois Roberts
Miss Mary Tavares

LIBRARY

Mrs. Virginia Brandenburg
Miss Elizabeth A. Brown
Mrs. Lenora Joseph
Albert K. Neal
Mrs. Doris T. Ricker

MAINTENANCE OF BUILDINGS AND GROUNDS

Eldon P. Allen
John T. Brady
Bernard F. Cavanaugh
Cecil Costa
John V. Day
Manuel P. Dutra
Richard E. Geggatt, Jr.
Donald B. Lehy
Ralph H. Lewis
Russell F. Lewis
Stephen A. Mills
Robert A. Serrano
Frederick E. Thrasher
Frederick E. Ward
Edward J. White

DEPARTMENT OF RESEARCH SERVICE

Gail M. Cavanaugh
Lowell V. Martin
Mrs. Juliette T. Morin
Frank E. Sylvia

SUPPLY DEPARTMENT

Donald P. Cheney
David H. Graham
Robert Hampton
Robert M. Hebden
Lewis M. Lawday
Robert O. Lehy
Miss Joyce B. Lima
Edward F. Matthews
Bruno F. Trapasso

DINING HALL AND HOUSING

Robert T. Martin, Manager, Food Service
Norman Michaud, Head Chef
Mrs. Elizabeth Kuil, Supervisor, Dining Room
Mrs. Ellen T. Nickelson, Supervisor, Dormitories
Alan G. Lunn, Supervisor, Cottage Colony

3. Investigators: Lillie, Grass and Rand Fellows; Students

Independent Investigators, 1968

Abbott, Bernard C., Head and Professor of Biophysics and Physiology, University of Illinois
Abrahamson, Edwin W., Professor of Chemistry, Case Western Reserve University
Adelman, William J., Jr., Professor of Physiology, University of Maryland School of Medicine
Allen, Robert D., Chairman and Professo. of Biological Sciences, State University of New York at Albany

Anderson, Everett, Professor of Zoology, University of Massachusetts, Amherst
April, Ernest W., Graduate Student, Columbia University, College of Physicians and Surgeons
Armstrong, Philip B., Professor of Anatomy, State University of New York, Upstate Medical Center
Arnold, John M., Assistant Professor of Cytology, University of Hawaii
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Kempton, Rudolf T., Emeritus Professor of Biology, Vassar College
Kennedy, Eugene P., Professor of Biochemistry, Harvard Medical School
Keosian, John, Professor of Biology, Rutgers – The State University
Leighton, Joseph, Professor of Pathology, University of Pittsburgh School of Medicine
Levinthal, Cyrus, Professor of Biophysics, Massachusetts Institute of Technology
Lineaweaver, Thomas H., Marine Biological Laboratory
Lochhead, John H., Professor of Zoology, University of Vermont
Marks, Paul A., Professor of Medicine, Columbia University
Morrison, Martin, Chairman, Department of Chemistry, St. Jude Children's Research Hospital
Nachmansohn, David, Emeritus Professor of Biochemistry, Columbia University, College of Physicians and Surgeons
Nasatir, Maimon, Chairman and Professor of Biology, The University of Toledo
Nason, Alvin, Professor of Biology, The Johns Hopkins University
Novikoff, Alex B., Research Professor of Pathology, Albert Einstein College of Medicine
Pearlstein, Robert M., Research Staff Member, Oak Ridge National Laboratory
Rose, Irwin A., Chairman of Biochemistry Division, Institute for Cancer Research, Philadelphia
Rosenkranz, Herbert S., Associate Professor of Microbiology, Columbia University
Roth, Jay S., Professor of Biochemistry, University of Connecticut
Rowland, Lewis P., Chairman and Professor of Neurology, University of Pennsylvania
Russell, Henry D., Marine Biological Laboratory
Sager, Ruth, Professor of Biological Sciences, Hunter College

SATCHELL, DAVID G., Research Associate, Columbia University, College of Physicians and Surgeons
SCHLESINGER, R. WALTER, Chairman of Microbiology, Rutgers–The State University
SCOTT, ALLAN, Chairman and Professor of Biology, Colby College
SHEDLOVSKY, THEODORE, Professor of Biophysics, The Rockefeller University
SONNENBLICK, B. P., Professor of Biology and Radiation Science, Rutgers–The State University
SPECTOR, ABRAHAM, Associate Professor, Columbia University
SPERELAKIS, NICK, Professor of Physiology, University of Virginia School of Medicine
STEFFAN, WALLACE A., Entomologist, Bishiop Museum, Hawaii
STEPHENSON, WILLIAM K., Chairman and Professor of Biology, Earlham College
STETTEN, DEWITT, JR., Dean and Professor of Experimental Medicine, Rutgers Medical School
STETTEN, MARJORIE R., Research Professor of Experimental Medicine, Rutgers Medical School
STRAUSS, ELLIOTT W., Associate Professor of Medical Science, Brown University
STROMINGER, JACK L., Professor of Biochemistry, Harvard University
WAINIO, WALTER, Chairman and Professor of Biochemistry, Rutgers – The State University
WAKSMAN, BYRON H., Chairman and Professor of Microbiology, Yale University
WHEELER, GEORGE E., Associate Professor of Biology, Brooklyn College
WHITING, ANNA R., Consultant, Oak Ridge National Laboratory
WICHTERMAN, RALPH, Professor of Biology, Temple University
WILSON, THOMAS HASTINGS, Associate Professor of Physiology, Harvard Medical School
WITTENBERG, JONATHAN B., Professor of Physiology, Albert Einstein College of Medicine
YNTEMA, CHESTER L., Professor of Anatomy, State University of New York, Upstate Medical Center
YORKE, ELLEN D., Research Associate, University of Maryland
ZACKS, SUMNER I., Associate Professor of Pathology, Pennsylvania Hospital
ZIPSER, DAVID, Assistant Professor of Molecular Biology, Columbia University

Students, 1968

All students listed completed the formal course program, June 17–July 27. Asterisk indicates students completing post-course research program, July 28–August 31.

ECOLOGY

*BELL, WAYNE HARRELL, Harvard University
CINTRON, GILBERTO, JR., University of Puerto Rico
CONWAY, JOAN ROSE, University of Massachusetts
*CROWE, JOHN HENRY, University of California, Riverside
*GERHART, DAVID Z., Lehigh University
*GILBERT, MARY ANN BEECHER, University of Massachusetts
*GILBERT, WILLIAM H., III, University of Massachusetts
*GLAUS, KAREN JANE, Yale University
LESLEY, DAVID EARL, Tulane University
*MAYNARD, NANCY GRAY, University of Miami, Institute of Marine Sciences
*NICOTRI, MARY ELIZABETH, University of Michigan
PIERCE, SIDNEY K., JR., Florida State University
REISA, JAMES JOSEPH, JR., Northwestern University
RUBINSTEIN, JEFFREY, Bates College
*SOUTHWICK, EDWARD E., Washington State University
TRUE, MERRILL ALLAN, Tulane University
WEST, RONALD ROBERT, University of Oklahoma
*YOUNG, LILY Y., Harvard University

EMBRYOLOGY

BARKLEY, DAVID S., Princeton University
*BERESFORD, GAIL, Washington University
*EROSHEVICH, KHRISTINE E., College of St. Mary of the Springs
*GREEP, NANCY CAROLE, University of Michigan

*Haraway, Donna Jeanne, Yale University
*Kankel, Douglas Ray, Brown University
Ling, Lee-Nien Lillian, Yale University
Linsenmayer, Thomas F., University of Virginia
*Massover, William H., University of Chicago
*Noden, Drew Morrison, Washington University
*Pederson, Mary Lee, University of Wisconsin
*Rosenthal, Marsha Gail, University of Pennsylvania
*Rubin, Leo, State University of New York at Albany
*Samols, David R., University of Chicago
Shifrin, Sidney, National Institutes of Health
*Suddith, Robert Louis, Indiana University
*Thomas, David Dwight, University of Hawaii
*Turner, Robert Scott, Jr., University of Oregon
*Van Essen, David C., Harvard University
*Walker, Susan Louise, The Johns Hopkins University

EXPERIMENTAL BOTANY

*Brooks, R. Clarks, University of Chicago
Buckner, Robin Y., University of Kentucky
Carroll, Jackie W., University of Alabama
*Clayton, Marilyn, Wheaton College
*Gardner, Gary M., Harvard University
*Goff, Robert W., University of Massachusetts
*Hinman, Norman D., University of Connecticut
*Jenike, Michael A., University of Massachusetts
*Linkins, Arthur E., University of Massachusetts
Monahan, Thomas J., University of Connecticut
Moreth, Clarice M. C., Nova University
Mullins, John Q., University of Tennessee
Pecora, Richard A., Kent State University
*Redemske, Joyce, University of Illinois
*Roux, Stanley J., Yale University

PHYSIOLOGY

*Abramson, Maxwell, Massachusetts General Hospital
*Berry, Robert Wayne, University of Oregon
*Frank, Eric, Harvard University
Freedman, Jeffrey Carl, University of Pennsylvania
*Hassell, John Robert, University of Connecticut
*Hitchcock, Sarah Ellen, Case Western Reserve University
*Hudspeth, Albert James, Harvard Medical School
Kawai, Masataka, Princeton University
Krisch, Henry M., The Johns Hopkins University
*Kronstadt, Michael, Columbia University
Laskowski, Michael B., University of Oklahoma Medical School
*Lazarow, Paul B., The Rockefeller University
*Lee, Yung-Keun, The Johns Hopkins University
*Linck, Richard Wayne, Brandeis University
Mancini, Particia Elena, Yale University
Mayor, Eugene P., Marquette University
Moran, George W., Princeton University
*Nicolson, Garth L., University of California, San Diego
Nissenbaum, Leonard, University of Oregon
*Plunkett, William K., University of Massachusetts
Rando, Robert Richard, Harvard University
*Regenstein, Joe Mac, Brandeis University

*Rose, Birgit, Columbia University, College of Physicians and Surgeons
*Rossetti, Giuseppe Plinio, Oregon State University
*Shaw, Carol Anne, Massachusetts Institute of Technology
*Sorenson, Martha M., University of Washington
*Stafford, Walter Flemming, III, University of Connecticut
*Trotter, Philip James, New England Institute for Medical Research
*Weintraub, Harold, University of Pennsylvania Medical School
Weiss, James Paul, University of Rochester
**Buday, Attila, University of Montreal, Canada
**Course Auditor

INVERTEBRATE ZOOLOGY

*Altman, Morris D., New York University
*Appelhof, Mary A., University of Iowa
Audette, Valerie Anne, University of Vermont
Brandon, Stacia Ruth, Mississippi State College for Women
Brothers, Edward Bruce, Brooklyn College
*Connell, Penny May, Tulane University
Diggins, Sister Kieran, Northwestern University
*Edds, Kenneth Tiffany, University of Rhode Island
Ernau, Mildred Claire, State University of New York at Albany
Gallucci, Betty Jane B., North Carolina State University
Gintzler, Alan Richard, Hunter College
Gleye, Linda Gibson, Sarah Lawrence College
*Halverson, Roger Conrad, University of California, Santa Barbara
Harman, Willard Nelson, Cornell University
Harper, Elvin, Massachusetts General Hospital
Hartzband, David J., Syracuse University
*Henderson, David J., University of Glasgow, Scotland
Holtzman, Seymour, Brooklyn College
Jablecki, Cynthia Grace, Mount Holyoke College
*Lounibos, Leon Philip, University of Notre Dame
*Lubchenco, Jane Ann, Colorado College
*Martin, Alice Nina, University of Massachusetts
*McFadien, Margaret Smith, West Chester State College
*Nichols, Thomas Richard, Brown University
*Nickerson, Richard Paul, Syracuse University
Oaks, John Adams, Tulane University
Ottesen, Eric Albert, Harvard Medical School
Resnick, Cecily Ann, University of Illinois
Rhodes, Edwin W., Jr., U. S. Department of Interior, Bureau of Commercial Fisheries
Richards, Anne Conway, Goucher College
Romano, Mary M., Goucher College
Scott, Dana M., Jr., Drew University
Sheldon, Robert Archer, University of Georgia
Sherman, Robert George, Michigan State University
Slade, John Downey, Oberlin College
Stiles, Edmund W., Oberlin College
Van Wie, Donald Gary, Harvard University
Weatherly, W. Paul, Yale University
Wood, Jackie Dale, University of Illinois

4. Fellowships and Scholarships, 1968

The Lucretia Crocker Scholarship:
Robert Clarke Brooks, Experimental Botany Course
Lily Y. Young, Marine Ecology Course
The James Watt Mavor Scholarship:
David J. Henderson, Invertebrate Zoology Course
The Gary N. Calkins Memorial Scholarship:
Roger C. Halverson, Invertebrate Zoology Course
The Turtox-Croasdale Scholarship:
Arthur E. Linkins, III, Experimental Botany Course

5. Training Program

Fertilization and Gamete Physiology Research Training Program

I. Instructors

Charles B. Metz, University of Miami, Program Chairman
C. R. Austin, Cambridge University, England
Gertrude W. Hinsch, University of Miami
Hans Linskens, University of Nijmegen, The Netherlands
Alberto Monroy, University of Palermo, Italy
Leonard Nelson, University of Toledo

II. Secretary

Laura Hoskin

III. Trainees

Angerer, Lynne Musgrave, The Johns Hopkins University
Angerer, Robert C., The Johns Hopkins University
Crandall, David E., University of Rhode Island
Firtel, Richard A., California Institute of Technology
Goudsmit, Esther M., The Johns Hopkins University
Hartley, David P., Duke University School of Medicine
Jeffrey, William., University of Iowa
Mirkes, Philip E., University of Michigan
Mowbray, Rodney C., Iowa State University
Repsis, Lynn C., Yale University
Schultz, Gilbert A., University of Calgary
Umpierre, Carmen C., Michigan State University
Urban, Paul, Yale University
Valleau, William, University of Maine
Verhey, Catherine, University of Illinois
Weitlauf, Harry M., University of Kansas Medical School
Wilson, Harold J., University of Arizona

IV. Lectures

F. Longo	The fine structure of pronuclear development and fusion in *Arbacia punctulata*
C. Dodson	Pollination in orchids
L. Machlis	Gamete and zygote chemotaxis in the watermold *Allomyces*
L. H. Kleinholz	Crustacean hormones
G. W. Hinsch	Reproduction in the spider crab *Libinia emarginata*
A. Blackler	Embryonic origin of amphibian gametes
L. D. Smith	The formation of primordial germ cells in amphibians
D. Epel	The enzymatic basis of cell adhesion in sea urchin embryos
R. Dippell	Development of basal bodies
R. Brinster	Energy metabolism in the preimplantation mammalian embryo

6. TABULAR VIEW OF ATTENDANCE, 1964–1968

	1964	*1965*	*1966*	*1967*	*1968*
INVESTIGATORS—TOTAL	512	572	555	590	528
Independent	273	284	287	313	281
Library Readers	47	62	77	78	76
Research Assistants	192	227	191	199	171
STUDENTS—TOTAL	126	128	126	132	122
Invertebrate Zoology	40	41	37	41	39
Embryology	20	20	22	20	20
Physiology	30	30	29	31	30
Botany	19	20	18	20	15
Ecology	17	17	20	20	18
TRAINEES—TOTAL	16	16	16	16	17
TOTAL ATTENDANCE	668	734	710	738	667
Less persons represented in two categories	7	4	0	4	7
	661	730	710	734	660
INSTITUTIONS REPRESENTED—TOTAL	140	218	198	177	169
FOREIGN INSTITUTIONS REPRESENTED	32	27	28	29	23

7. INSTITUTIONS REPRESENTED, 1968

Alabama, University of
Albert Einstein College of Medicine
American Medical Association
American Museum of Natural History
Amherst College
Argonne National Laboratory
Arizona, University of
Bates College
Battelle Memorial Institute
Bell Telephone Laboratories
Bishop Museum, Honolulu
Boston University
Brandeis University
Brookhaven National Laboratory
Brooklyn College
Brown University
California Institute of Technology
California, University of, Davis
California, University of, Los Angeles
California, University of, Riverside
California, University of, San Diego
California, University of, San Francisco
California, University of, Santa Cruz
Case Western Reserve University
Chicago, University of
Cincinnati, University of
City College of New York
Clapp, W. F., Laboratories
Colby College
College of St. Mary of the Springs
Colorado College
Colorado, University of, Medical Center
Columbia University
Columbia University, College of Physicians and Surgeons
Connecticut, University of
Connecticut, University of, Medical School
Cornell University
Cornell University Medical College, New York City
Dartmouth College
Dartmouth Medical School
Davidson College
Delaware, University of
Denison University
Drew University
Duke University
Duke University Medical School
Earlham College
Emory University
Florida Oceanographic Society
Florida State University
Georgia, University of
Goucher College
Hamilton College
Harvard Medical School
Harvard University
Hawaii, University of
Hunter College
Illinois Institute of Technology
Illinois, University of
Indiana University

Indiana University Medical School
Institute of Biomedical Research, Chicago
Institute for Cancer Research, Philadelphia
Institute of Molecular Evolution, Coral Gables
Institute for Muscle Research, Woods Hole
Iowa State University
Iowa, University of
John Carroll University
Johns Hopkins Hospital, The
Johns Hopkins University, The
Johns Hopkins University, The, School of Hygiene and Public Health
Johns Hopkins University, The, School of Medicine
Kansas, University of
Kansas, University of, Medical Center
Kent State University
Kentucky, University of
Kenyon College
Lehigh University
Marine Biological Laboratory
Marquette University
Maryland, University of, School of Medicine
Massachusetts General Hospital
Massachusetts Institute of Technology
Massachusetts, University of
Medical College of Ohio at Toledo
Mellon Institute of Carnegie-Mellon University
Miami, University of
Miami, University of, Institute of Marine Sciences
Miami University, Oxford, Ohio
Michigan State University
Michigan, University of
Minnesota, University of
Mississippi State College for Women
Mount Holyoke College
National Institutes of Health
New England Institute for Medical Research
New Mexico, University of, School of Medicine
New York State Institute for Basic Research in Mental Retardation
New York University
New York University College of Dentistry
New York University Medical School
New York University, Washington Square College of Arts and Sciences
North Carolina, University of
North Carolina State University, Raleigh
North Dakota, University of
Northeastern Illinois State College
Northwestern University
Notre Dame, University of
Nova University
Oak Ridge National Laboratory
Oberlin College
Ohio Dominican College
Oklahoma, University of
Oregon State University
Oregon, University of
Pennslyvania Hospital
Pennslyvania, University of
Pennslyvania, University of, School of Medicine
Peter Bent Brigham Hospital
Pittsburgh, University of, School of Medicine
Princeton University
Providence College
Puerto Rico, University of
Queens College of the City University of New York
Radcliffe College
Reed College
Rensselaer Polytechnic Institute
Rhode Island, University of
Rice University
Rochester, University of
Rochester, University of, School of Medicine and Dentistry
Rockefeller University, The
Russell Sage College
Rutgers Medical School
Rutgers–The State University
St. Jude Children's Research Hospital
Saint Louis University
Sarah Lawrence College
Scripps Institute of Oceanography
Single Cell Research Foundation
Sloan-Kettering Institute for Cancer Research
Smith College
Smithsonian Institution
Stanford University
State University of New York, Albany
State University of New York, Buffalo
State University of New York, Syracuse
State University of New York, Downstate Medical Center
State University of New York, Upstate Medical Center
Syracuse University
Temple University
Texas, University of, Medical School
Toledo, University of
Trinity College
Tulane University
Union College
U. S. Department of Interior, Bureau of Commercial Fisheries
Vassar College
Vermont, University of
Veterans Administration Central Office
Veterans Administration Hospital, Albany
Veterans Administration Hospital, Brooklyn
Virginia, University of
Virginia, University of, School of Medicine
Washington University
Washington University School of Medicine

West Chester State College
West Virginia University
Wheaton College
Wilson College
Wisconsin, University of
Woods Hole Oceanographic Institution
Yale University
Yale University School of Medicine

Foreign Institutions Represented, 1968

Biologische Anstalt Helgoland, West Germany
Bristol, University of, England
Calgary, University of, Canada
Cambridge, University of, England
Dalhousie University, Canada
Glasgow, University of, Scotland
Hebrew University of Jerusalem, Israel
Hiroshima University, Japan
Instituto Venezolano de Investigaciones Cientificas, Venezuela
King's College, University of London, England
London, University of, England
MRC Laboratory of Molecular Biology, England
Marine Science Laboratories, Wales
McGill University, Canada
Middlesex Hospital Medical School, England
Montreal, University of, Canada
Nijmegen University, The Netherlands
Ottawa, University of, Canada
Palermo, University of, Italy
Paris, University of, France
Tokyo Dental College, Japan
Zoologische Anstalt Basel, Switzerland

Supporting Institutions, Agencies, and Individuals

Associates of the Marine Biological Laboratory
Atomic Energy Commission
Mr. and Mrs. W. Van Alan Clark
Clowes Fund
Kenneth S. Cole
Mrs. W. Murray Crane
Dr. William D. Curtis
The Ford Foundation
Mr. W. T. Golden
The Grass Foundation
Mrs. James W. Mavor
National Institutes of Health
National Science Foundation
Northfield Mines, Inc.
Office of Naval Research
Mrs. Arthur Kemble Parpart
Mr. and Mrs. Gerard Swope, Jr.
Whitehall Foundation, Inc.

8. Friday Evening Lectures, 1968

June 28

Ernest Mayr .... The study of biological diversity
The Agassiz Museum
Harvard University

July 5

Emanuel Margoliash .... Evolutionary variability of cytochrome C
Abbott Laboratories, Chicago

July 11

Sir John Eccles .... Postsynaptic inhibitory pathways of the central nervous system
Institute for Biomedical Research,
Alexander Forbes Lecturer at the
Marine Biological Laboratory

July 12

Sir John Eccles .... Alexander Forbes—Neurophysiologist

July 12

Sir John Eccles .... Integrative action of the nervous system with special reference to the cerebellum

July 19	
Edward O. Wilson Harvard University	Theory and experiment in zoögeography
July 26	
Lewis Wolpert Middlesex Hospital Medical School, Lillie Fellow at the Marine Biological Laboratory	The cell in morphogenesis and pattern formation
August 2	
Gerald Elliott Kings' College, London, Rand Fellow at the Marine Biological Laboratory	The muscle fiber—A lyotrophic liquid crystal
August 9	
Roger Stanier University of California, Berkeley	Evolutionary significance of regulatory mechanisms
August 16	
H. Keffer Hartline The Rockefeller University	Visual receptors and retinal interaction
August 23	
Seymour S. Cohen University of Pennsylvania School of Medicine	Sponges, comparative biochemistry and cancer chemotherapy

9. Tuesday Evening Seminars, 1968

July 16	
Seymour Zigman	Sulfonation of rat lens proteins
Audrey E. V. Haschemeyer	Possible role of protein synthetic enzymes in the control of protein synthesis in higher organisms
James R. Bolton Roderick K. Clayton	Quantum efficiency of the light-induced bleaching of P870 in *Rhodopseudomonas spheroides* reaction centers
July 23	
Robert B. Hill	The relationship between carbon atom chain length and effectiveness of n-alkylamines in inducing rhythmicity of gastropod smooth muscle
K. Ranga Rao Milton Fingerman	Dimorphic variation of pigmentary patterns in the fiddler crab, *Uca pugilator*
Daniel S. Grosch	Reproductive performance of insects from U. S. Biosatellite II
July 30	
Albert Szent-Györgyi	Regulation of cell division
John M. Arnold	Formation of the first cleavage furrow in a telolecithal egg (*Loligo*)

Roger Milkman	On the distribution of genetic variation in nature
Robert K. Josephson Hugh Y. Elder	Rapidly contracting muscles used in sound production by a katydid

August 6

C. Lloyd Claff Armand Crescenzi Arnold Landé	Shark and dogfish studies: Pulsatile pressure extracorporeal blood oxygenation by means of artificial silicone membrane heart-lung devices and artificial kidney
John L. Roberts	Thermal stress and gill ventilation by fishes
Katharine D. Hobson	A sexual reproduction of *Pygospio elegans* Claparède in Barnstable Harbor, Massachusetts
Marc H. Dresden	Limb regeneration in adult amphibians

August 13

J. Metuzals	Configuration of a filamentous network in the axoplasm of the squid giant nerve fiber
David Shemin	Effect of K^+ ions on the conformation of an enzyme
Milton Levy	The unreasonably high molar activity of nerve growth factor
Rev. J. C. Cassidy, O. P. L. J. Beauregard C. F. Starmer	Quantitative radioautography of vitellogenesis in *Artemia salina*

10. Members of the Corporation, 1968

Including Action of 1968 Annual Meeting

Life Members

Adolph, Dr. Edward F., University of Rochester School of Medicine and Dentistry, Rochester, New York 14620
Baitsell, Dr. George A., Winter Park Tower, Winter Park, Florida 32789
Brodie, Mr. Donald, 522 Fifth Avenue, New York, New York 10018
Cole, Dr. Elbert C., 2 Chipman Park, Middlebury, Vermont 05753
Cowdry, Dr. E. V., 4580 Scott Avenue, St. Louis, Missouri 63110
Crane, Mrs. W. Murray, 820 Fifth Avenue, New York, New York 10021
Curtis, Dr. Maynie R., Cancer Research Laboratory, School of Medicine, University of Miami, Coral Gables, Florida 33146
Dawson, Dr. A. B., 12 Scott Street, Cambridge, Massachusetts 02138
Dawson, Dr. J. A., 129 Violet Avenue, Floral Park, Long Island, New York 11001
Hess, Dr. Walter, 787 Maple Street, Spartanburg, South Carolina 29302
Hisaw, Dr. F. L., Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138
Irving, Dr. Laurence, University of Alaska, College, Alaska 99735
Jacobs, Dr. M. H., Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104
Lowther, Dr. Florence, Barnard College, New York, New York 10027
MacDougall, Dr. Mary Stuart, Mt. Vernon Apartments, 423 Clairmont Avenue, Decatur, Georgia 30030

Malone, Dr. E. F., 6610 North 11th Street, Philadelphia, Pennsylvania 19126
Means, Dr. J. H., 15 Chestnut Street, Boston, Massachusetts 02108
Medes, Dr. Grace, 303 Abington Avenue, Philadelphia, Pennsylvania 19111
Payne, Dr. Fernandus, Indiana University, Bloomington, Indiana 47405
Plough, Dr. H. H., 15 Middle Street, Rt. 1, Amherst, Massachusetts 01002
Pond, Samuel E., 53 Alexander Street, Manchester, Connecticut 06040
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania 19104
Schrader, Dr. Sally, Duke University, Durham, North Carolina 27706
Smith, Dr. Dietrich C., 218 Oak Street, Catonsville, Maryland 12128
Straus, Dr. W. L., Jr., Department of Anatomy, The Johns Hopkins University Medical School, Baltimore, Maryland 21205
Turner, Dr. C. L., Northwestern University, Evanston, Illinois 60201
Waite, Dr. F. G., 144 Locust Street, Dover, New Hampshire 03820
Wallace, Dr. Louise B., 359 Lytton Avenue, Palo Alto, California 94301
Warren, Dr. Herbert S., 2768 Egypt Road, Audubon, Pennsylvania 19407

Regular Members

Abbott, Dr. Bernard C., Department of Biological Sciences, University of Southern California, University Park, Los Angeles, California 90007
Adelberg, Dr. Edward A., Department of Microbiology, Yale University, New Haven, Connecticut 06510
Adelman, Dr. Wm. J., Jr., Department of Physiology, University of Maryland, Medical School, Baltimore, Maryland 21201
Allen, Dr. M. Jean, Department of Biology, Wilson College, Chambersburg, Pennsylvania 17201
Allen, Dr. Robert D., Department of Biological Sciences, State University of New York, Albany, New York 12203
Alscher, Dr. Ruth, Department of Biology, Manhattanville College, Purchase, New York 10577
Amatniek, Mr. Ernest, 34 Horner Avenue, Hastings-on-the-Hudson, New York 10706
Amerson, Dr. William R., Katy Hatch Road, Falmouth, Massachusetts 02540
Anderson, Dr. Everett, Department of Zoology, University of Massachusetts, Amherst, Massachusetts 01003
Anderson, Dr. J. M., Division of Biological Sciences, Stimson Hall, Cornell University, Ithaca, New York 14850
Anderson, Dr. Rubert S., Box 113, Woods Hole, Massachusetts 02543
Armstrong, Dr. Philip B., Department of Anatomy, State University of New York, College of Medicine, Syracuse, New York 13210
Arnold, Dr. John Miller, P.B.R.C., 2538 The Mall, University of Hawaii, Honolulu, Hawaii 96822
Arnold, Dr. William A., Division of Biology, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830
Ashworth, Dr. John Michael, Department of Biochemistry, Leicester University, Leicester, England

ATWOOD, DR. KIMBALL C., Department of Microbiology, University of Illinois, Urbana, Illinois 61801

AUCLAIR, DR. WALTER, Department of Biology, Rensselaer Polytechnic Institute, Troy, New York 12181

AUSTIN, DR. COLIN RUSSELL, Physiological Laboratory, Cambridge University, Downing Street, Cambridge, England

AUSTIN, DR. MARY L., 506½ North Indiana Avenue, Bloomington, Indiana 47401

BACON, MR. ROBERT, Church Street, Woods Hole, Massachusetts 02543

BAKALAR, MR. DAVID, 330 Beacon Street, Boston, Massachusetts 02167

BALL, DR. ERIC G., Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

BALLARD, DR. WILLIAM W., Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755

BANG, DR. F. B., Department of Pathobiology, The Johns Hopkins University School of Hygiene, Baltimore, Maryland 21205

BARD, DR. PHILLIP, The Johns Hopkins Medical School, Baltimore, Maryland 21205

BARTH, DR. L. G., Marine Biological Laboratory, Woods Hole, Massachusetts 02543

BARTH, DR. LUCENA, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

BARTLETT, DR. JAMES H., Department of Physics, University of Alabama, P. O. Box 1921, University, Alabama 35486

BAUER, DR. G. ERIC, Department of Anatomy, University of Minnesota, Minneapolis, Minnesota 55414

BAYLOR, DR. E. R., State University of New York, Stony Brook, Long Island, New York 11790

BAYLOR, DR. MARTHA A., State University of New York, Stony Brook, Long Island, New York 11790

BEAMS, DR. HAROLD W., Department of Zoology, State University of Iowa, Iowa City, Iowa 52240

BECK, DR. L. V., Department of Pharmacology, Indiana University, School of Experimental Medicine, Bloomington, Indiana 47401

BEHRE, DR. ELINOR M., Black Mountain, North Carolina 28711

BELAMARICH, DR. FRANK A., Department of Biology, Boston University, Boston, Massachusetts 02215

BELL, DR. ALLEN, Department of Anatomy, University of Colorado, Medical Center, Denver, Colorado 80220

BELL, DR. EUGENE, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

BENNETT, DR. MICHAEL V. L., Department of Anatomy, Albert Einstein College of Medicine, Bronx, New York 10461

BENNETT, DR. MIRIAM F., Department of Biology, Sweet Briar College, Sweet Briar, Virginia 24595

BERG, DR. WILLIAM E., Department of Zoology, University of California, Berkeley, California 94720

BERMAN, DR. MONES, National Institutes of Health, Institute for Arthritis and Metabolic Diseases, Bethesda, Maryland 20014

Berne, Dr. Robert M., University of Virginia School of Medicine, Charlottesville, Virginia 22903

Bernheimer, Dr. Alan W., New York University College of Medicine, New York, New York 10016

Bernstein, Dr. Maurice, Department of Anatomy, Wayne State University College of Medicine, Detroit, Michigan 48237

Bersohn, Dr. Richard, Department of Chemistry, Columbia University, 959 Havemeyer Hall, New York, New York 10027

Bertholf, Dr. Lloyd M., Central States College Association, 2530 Crawford Avenue, Evanston, Illinois 60201

Bevelander, Dr. Gerrit, Dental Branch, Medical Center, University of Texas, Houston, Texas 77025

Bigelow, Dr. Henry B., Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts 02138

Biggers, Dr. John Dennis, The Johns Hopkins University School of Hygiene and Public Health, Division of Population Dynamics, Baltimore, Maryland 21205

Bishop, Dr. David W. Department of Animal Science, Morrison Hall, Cornell University, Ithaca, New York 14850

Blanchard, Dr. K. C., The Johns Hopkins University Medical School, Baltimore, Maryland 21205

Bloch, Dr. Robert, Adalbertstr. 70, 8 Munich, 13 Germany

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Mast, Mrs. S. O.
Mather, Mr. Frank J., III
Mavor, Mrs. James W., Sr.
McCusker, Mr. and Mrs. Paul T.
McElroy, Mrs. Nella W.
McGillicuddy, Dr. and Mrs. John J.
McLane, Mrs. Huntington
Meigs, Mr. and Mrs. Arthur
Meigs, Dr. and Mrs. J. Wister
Metz, Mrs. Charles B.
Mitchell, Mrs. Philip
Mixter, Mrs. W. I.
Montgomery, Dr. and Mrs. Charles H.
Morse, Mr. and Mrs. Richard S.
Naterman, Dr. and Mrs. Hyman L.
Newton, Miss Helen K.
Nichols, Mrs. George
Nickerson, Mr. and Mrs. Frank L.
Norman, Mr. and Mrs. Andrew
Normandie Foundation Inc.
Packard, Mrs. Charles
Park, Mr. and Mrs. Franklin A.
Park, Mr. Malcolm S.
Parpart, Mrs. Arthur K.
Patten, Mrs. Bradley
Pendleton, Dr. Murray E.
Pennington, Miss Anne H.
Perkins, Mr. and Mrs. Courtland D.
Philippe, Mr. and Mrs. Pierre
Putnam, Mr. and Mrs. William A., III
Ratcliffe, Mr. Thomas, Jr.
Redfield, Dr. and Mrs. Alfred
Reznikoff, Dr. and Mrs. Paul
Riggs, Mr. and Mrs. Lawrason, III
Robertson, Dr. and Mrs. C. W.
Robinson, Mr. Denis M.
Rogers, Mrs. Charles E.
Root, Dr. and Mrs. Walter S.
Rugh, Dr. and Mrs. Roberts
Saunders, Mr. and Mrs. Lawrence
Schwartz, Mrs. Victor A.
Shiverick, Mrs. Arthur
Smith, Mrs. Homer P.
Speidel, Mrs. Carl C.
Steinbach, Mrs. H. Burr
Stetten, Dr. and Mrs. DeWitt, Jr.
Stone, Mr. and Mrs. Leo
Stone, Mrs. Samuel M.
Stunkard, Dr. Horace W.
Swanson, Mrs. Carl P.
Swope, Mr. and Mrs. Gerard, Jr.
Swope, Mr. and Mrs. Gerard L.
Swope, Miss Henrietta H.

Tompkins, Mr. and Mrs. B. A.
Waksman, Dr. and Mrs. Byron H.
Waksman, Dr. and Mrs. Selman A.
Wallace, Dr. and Mrs. Stanley L.
Wang, Dr. and Mrs. An
Ware, Mr. and Mrs. J. Lindsay
Warren, Dr. and Mrs. Shields
Watt, Mr. and Mrs. John B.
Webster, Mrs. Edwin S.
Whiteley, Mr. and Mrs. George C., Jr.
Whiting, Dr. and Mrs. Phineas W.
Whitney, Mr. G. G., Jr.
Wichterman, Mrs. Ralph
Wickersham, Mrs. James H.
Wilhelm, Mr. and Mrs. Hilmar J.
Wilson, Mrs. Edmund B.
Wilson, Dr. May G.
Witmer, Dr. and Mrs. Enos
Wolfe, Dr. Charles
Wolfinsohn, Mrs. Wolfe
Wrinch, Dr. Dorothy
Yntema, Mrs. Chester L.
Zwilling, Mrs. Edgar

V. REPORT OF THE LIBRARIAN

The first annual report, 80 years ago, stated:

> "A library is a necessity and it must be provided for in one way or another. Boston libraries are near enough to be of great service, but we cannot depend on them alone . . . we must have, as the minimum to begin with, all the important journals now printed in the four principal languages. It is most earnestly to be hoped that adequate means may be found to meet this all important requisite."

Adequate means were found. Today over 4,000 separate journal titles, in 30 languages, totaling 136,561 volumes are contained in the collection at Woods Hole. Boston libraries, as well as numerous libraries throughout the country and abroad depend on the Laboratory's library for service. In 1948 outside requests for journal articles totaled 180. This year we received 4,532 individual requests. As new colleges, industrial firms, and research laboratories are established, the MBL collection, 80 years in building, becomes increasingly valuable.

One of the main concerns of recent library committees has been the need for more space. Approximately 2,500 bound volumes and 1,000 unbound publications were added to the stacks annually and the journal sections of the library are now about to burst and pour down to the reprint floor. The committee recommended that the reprints should either be eliminated or removed to another area. A written vote of the members of the corporation at the annual meeting showed that the reprints must *not* be eliminated. Therefore a new location for this collection, totalling 243,000 reprints and covering one entire floor of the library, must be found.

Twenty years ago the library held 57,548 volumes. Today the total is 136,561.

Total number of journal titles received yearly in 1948 was 1,209. In 1968 the number received currently was 2,335.

Binding expenses in 1948 were $1,852. This year the library binding costs were $8,329.

In the past 20 years 100,000 reprints have been added to the reprint floor.

Twenty years ago the Woods Hole Oceanographic Institution budgeted $800 for the purchase of oceanographic material to be added to the collection. Today they are budgeting $15,000. Fortunately the Oceanographic has been adding their literature to the library since they were founded in 1930. The combined collection has led many to say that the most complete biological and oceanographical library in the world is here in Woods Hole.

If the MBL library is to continue to be of great service to the scientific community all new programs and techniques of information retrieval must be considered. The rapid development of xerography opens many possibilities. Future scientists may wish to purchase rare books individually rather than borrow, and the new technology may offer devices whereby this would not only be feasible but practical. This coming summer the library committee is planning working displays of computer retrieval equipment and hopefully many scientists will be drawn into discussion as to what they will want and expect of the MBL library in the years ahead.

VI. REPORT OF THE TREASURER

The market value of the General Endowment Fund and the Library Fund at December 31, 1968, amounted to $2,403,484 and the corresponding securities are entered in the books at a value of $1,575,096. This compares with values of $2,313,618 and $1,397,833 respectively, at the end of the preceding year. The average yield on the securities was 3.60% of the market value and 5.49% of the book value. Uninvested principal cash was $624. Classification of the securities held in the Endowment Fund appears in the Auditor's Summary of Investments.

The market value of the Pooled Securities at December 31, 1968, amounted to $824,502 as compared to book values of $666,202. These figures compare with values of $710,152 and $618,416, respectively, at the close of the preceding year. The average yield on the securities was 3.26% of the market value and 4.04% of the book value. Uninvested principal cash was in the amount of $7,653.

The proportionate interest in the Pool Fund Account of the various funds, as of December 31, 1968, is as follows:

Pension Funds	22.492%
General Laboratory Investment	22.446%
F. R. Lillie Memorial Fund	2.447%
Anonymous Gift	.839%
Other:	
Bio Club Scholarship Fund	.638%
Rev. Arsenius Boyer Scholarship Fund	.772%
Gary N. Calkins Fund	.728%
Allen R. Memhard Fund	.142%
Lucretia Crocker Fund	2.649%
E. G. Conklin Fund	.446%
Jewett Memorial Fund	.236%
M. H. Jacobs Scholarship Fund	.319%
Herbert W. Rand Fellowship	22.637%
Mellon Foundation	10.685%
Mary Rogick Fund	2.342%
Swope Foundation	5.879%
Clowes Fund	4.303%

Donations from MBL Associates for 1968 amounted to $8,360 as compared with $8,010 for 1967. Unrestricted gifts from foundations, societies and companies amounted to $1,378.

During the year we administered the following grants and contracts:

Investigators	*Training*	*MBL Institutional*
7 NIH	3	2 NIH
5 NSF	1	3 NSF
1 Ford		1 Ford
1 Whitehall		1 AEC
1 ONR		1 ONR
15	4	8

The rate of overhead on continuing grants from Federal agencies is effectively 20% of the amounts expended. For new Federally funded research grants a provisional rate of 25% has been negotiated. However, the Laboratory is required to make an identifiable contribution to the total cost of each research project. The overhead on those grants wherein therefore was made in the funds awarded within the year, amounted to $96,482 as compared with $95,874 for the preceding year. A proposal to NSF for determining an indirect cost rate for 1968 and 1969 is under consideration.

The following is a statement of the auditors:

To the Trustees of Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheet of Marine Biological Laboratory as at December 31, 1968, the related statement of operating expenditures and income and statement of funds for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We examined and have reported on financial statements of the Laboratory of the year ended December 31, 1967.

In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of Marine Biological Laboratory at December 31, 1968 and 1967 and the results of its operations for the years then ended on a consistent basis.

The supplementary schedules included in this report were obtained from the Laboratory's records in the course of our examination and, in our opinion, are fairly stated in all material respects to the financial statements, taken as a whole.

Boston, Massachusetts
March 29, 1969 LYBRAND, ROSS BROS. AND MONTGOMERY

It will be noted from the operating statement that the Laboratory activities for the year under review, amounted to a figure of a little over 1.5 million dollars, which amount is comparable to last years results.

ALEXANDER T. DAIGNAULT,
Treasurer

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1968 and 1967

Investments

	1968	*1967*
Investments held by Trustee:		
Securities, at cost (approximate market quotation1968—$2,403,484)	$1,575,096	$1,397,833
Cash	624	4,728
	1,575,720	1,402,561
Investments of other endowment and unrestricted funds:		
Pooled investments, at cost (approximate market quotation 1968—$824,502) less $5,728 temporary investment of current fund cash	660,474	612,688
Other investments	2,282,150	2,803,850
Cash	7,653	387
Accounts receivable	33	54
Due from current fund	53,276	87,542
	$4,579,306	$4,907,082

Plant Assets

Land, buildings, library and equipment (note)	5,732,782	5,695,732
Less allowance for depreciation (note)	1,711,311	1,624,698
	4,021,471	4,071,034
Construction in progress	1,316,468	110,042
Investments at cost which approximates market	842,629	50,000
Due from current funds	38,531	15,365
	$6,219,099	$4,246,441

Current Assets

Cash	251,061	107,483
Temporary investment in pooled securities	5,728	5,728
Accounts receivable (U. S. Government, 1968—$33,824; 1967—$83,619)	130,503	139,771
Inventories of supplies and bulletins	47,036	46,372
Other assets	7,146	8,698
Due to plant funds	(38,531)	(15,365)
Due to endowment funds	(53,276)	(87,542)
	$ 349,667	$ 205,145

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1968 and 1967

Invested Funds

	1968	*1967*
Endowment funds given in trust for benefit of the Marine Biological Laboratory	$1,575,720	$1,402,561
Endowment funds for awards and scholarships:		
Principal	427,663	427,663
Unexpended income	39,394	32,738
	467,057	460,401
Unrestricted funds functioning as endowment	2,336,190	2,857,890
Retirement fund	192,543	169,833
Pooled investments—accumulated gain	7,796	16,397
	$4,579,306	$4,907,082

Plant Funds

Funds expended for plant, less retirements	6,886,046	5,805,774
Less allowance for depreciation charged thereto	1,711,311	1,624,698
	5,174,735	4,181,076
Accounts payable	163,204	
Unexpended plant funds	881,160	65,365
	$6,219,099	$4,246,441

Current Liabilities and Funds

Accounts payable and accrued expenses	44,737	1,947
Advance subscriptions	23,712	15,210
Unexpended grants—research	25,727	32,353
Unexpended balances of gifts for designated purposes	23,764	23,899
Current fund	231,727	131,736
	$ 349,667	$ 205,145

Note—The Laboratory has since January 1, 1916 provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

STATEMENT OF OPERATING EXPENDITURES AND INCOME

Years Ended December 31, 1968 and 1967

	1968						
	Salaries and Wages	*Other Costs and Expenses*	*Depreciation*	*Total*	*Charged to Grants*	*Total*	*1967*
Operating Expenditures							
Instruction		$ 13,160	$ 8,905	$ 22,065	$195,800	$ 217,865	$ 209,549
Research		29,810	59,596	89,406	416,231	505,637	527,492
Dormitories	$ 19,932	37,929	11,881	69,742		69,742	65,500
Dining	21,188	39,000	1,674	61,862		61,862	55,892
Library	30,430	11,470		41,900		41,900	39,338
Back sets, serials and binding		37,051		37,051		37,051	34,626
Biological Bulletin	3,231	36,988		40,219		40,219	28,370
Support services:							
Apparatus	47,042	41,172		88,214	9,484	97,698	100,664
Supply	84,600	52,587	4,557	141,744		141,744	135,106
Administration	81,338	65,936		147,274		147,274	152,419
Plant operation	127,089	90,778		217,867		217,867	221,050
Grant expenditures for support services					43,584	43,584	89,228
Other		38,082		38,082		38,082	26,981
	$414,850	$493,963	$ 86,613	995,426	$665,099	1,660,525	1,686,215

MARINE BIOLOGICAL LABORATORY

Statement of Operating Expenditures and Income

Years Ended December 31, 1968 and 1967

	Fees	*Other*	*Total*	*Charged to Grants*	*Total*	*1967*
Income						
Instruction	37,680		37,680	195,800	233,480	226,747
Research	128,415		128,415	416,231	544,646	548,022
Dormitories		82,833	82,833		82,833	72,007
Dining		73,135	73,135		73,135	69,210
Library	20,391	26,228	46,619		46,619	36,688
Biological Bulletin	38,451		38,451		38,451	38,119
Support services:						
Apparatus		31,409	31,409		31,409	33,833
Supply		45,602	45,602		45,602	42,247
Administration		12,769	12,769		12,769	11,909
Investments income		244,364	244,364		244,364	180,954
Gifts used for current expense		13,224	13,224		13,224	27,868
Allowance for indirect costs		96,482	96,482		96,482	95,874
Grants for general support		78,160	78,160	9,484	87,644	132,097
Grants for support services				43,584	43,584	89,228
Other		3,212	3,212		3,212	8,085
	$224,937	$707,418	932,355	$665,099	1,597,454	1,612,888
Excess of current expenditures and depreciation over current income			63,071		63,071	73,327
Reduction in plant funds for depreciation			86,613		86,613	87,226
Excess current income			$ 23,542		$ 23,542	$ 13,899

MARINE BIOLOGICAL LABORATORY

STATEMENT OF FUNDS

Year Ended December 31, 1968

	Balance December 31, 1967	*Gifts and Other Receipts*	*Investment Income*	*Used for Current Expenses*	*Other Expenditures*	*Balance December 31, 1968*
Invested funds	$4,907,082	$ 194,483	$265.049	$ 244,123	$ 543,185	$4,579,306
Unexpended plant funds	$ 65,365	812,396	3,399			$ 881,160
Unexpended research grants	$ 32,353	1,436,906		839,358	604,174	$ 25,727
Unexpended gifts for designated purposes	$ 23,899	9,126		8,360	901	$ 23,764
Current fund	$ 131,736	23,542 (1) (30,227) (2) 521,700 (3) (415,024) (4)				$ 231,727
		$2,552,902	$268,448	$1,091,841	$1,148,260	
Gifts		821,522				
Grant for facilities construction		604,174				
Grants for research, training and support		832,732				
Appropriated from current income and other		29,925				
Net gain on sale of securities		164,558				
(1) Excess of current income over expenditures		23,542				
(2) Adjustment of prior years' support grants		(30,227)				
(3) Transfer from invested funds		521,700				
(4) Expended for new laboratory and dormitory		(415,024)				
		$2,552,902				
Expended for new laboratory and dormitory					1,019,198	
Scholarship awards					8,025	
Payments to pensioners					13,417	
Expended for current operations					106,676	
Other					944	
					$1,148,260	

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1968

	Cost	*% of Total*	*Market Quotations*	*% of Total*	*Investment Income 1968*
Securities held by Trustee:					
General endowment fund:					
U. S. Government securities......	$ 25,065	2.0	$ 24,360	1.2	$ 1,250
Corporate bonds.................	712,367	57.4	595,270	30.4	30,256
Preferred stocks.................	113,044	9.1	155,657	7.9	4,142
Common stocks.................	390,974	31.5	1,185,185	60.5	36,216
	1,241,450	100.0	1,960,472	100.0	71.864
General educational board endowment fund:					
U. S. Government securities......	53,112	15.9	51,642	11.7	2,650
Other bonds....................	184,745	55.4	157,965	35.7	6,909
Preferred stocks.................	47,323	14.2	83,480	18.8	980
Common stocks.................	48,466	14.5	149,925	33.8	4,117
	333,646	100.0	443,012	100.0	14,656
Total securities held by Trustee	$1,575,096		$2,403,484		86,520
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government securities......	13,795	2.1	14,000	1.7	765
Corporate bonds.................	217,194	32.6	199,395	24.2	10,343
Common stocks.................	435,213	65.3	611,107	74.1	15,792
	666,202	100.0	$ 824,502	100.0	26,900
Less temporary investment of current fund cash...........	(5,728)				(241)
	660,474				26,659
Other investments:					
U. S. Government securities......	27,938				1,133
Other bonds....................	15,029				750
Preferred stocks.................	3,448				135
Common stocks.................	46,186				2,483
Real estate.....................	17,549				
Short-term commercial notes......	2,172,000				146,074
	2,282,150				150,575
Total investments of other endowment and unrestricted funds.......	$2,942,624				177,234
Total................................					263,754

	Cost	*Investment Income 1968*
Custodian's fees charged thereto		(3,080)
Adjustment of prior years' income		4,375
Investment income distributed to invested funds		265,049
Plant investments:		
Federal agency and corporate bonds	340,000	3,399
Common stock	502,629	
	$ 842,629	
Current investments:		
Temporary investment in pooled securities	$ 5,728	241
Total investment income		$268,689

Reference: *Biol. Bull.*, **137**: 79–94. (August, 1969)

THE INFLUENCE OF LIGHT ON ASEXUAL REPRODUCTION IN GREEN AND APOSYMBIOTIC HYDRA

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The occurrence of symbiotic algae in certain hydra has been known since the description of the first green hydra from southern Russia by Peter Simon Pallas over 200 years ago, but despite numerous studies on green hydra and its algae relatively little is known about the nature of the relationship between the algae and their hosts.

Early studies on the nature of this relationship suggested some contribution of the algae to the nutrition of the hydra, but were carried out with crude culture conditions and generally lacked quantitation (*e.g.*, Whitney, 1907, 1908; Goetsch, 1924; and Haffner, 1925). Recent studies using more effectively controlled culture conditions and improved quantitative techniques have provided more definite evidence of algal contribution to the nutrition of the host hydra (Muscatine, 1965; Muscatine and Lenhoff, 1963, 1965; Muscatine, Karakashian and Karakashian, 1967; Stiven, 1965; and Park, Greenblatt, Mattern and Merrill, 1967). All of these investigations have indicated that the algae in green hydra make no significant contribution to the nutrition of well-fed animals. Most have further indicated that under conditions of starvation the algae may make a positive contribution to the nutrition of the host, although two of the reports (Whitney, 1907; and Park *et al.*, 1967) have shown that green hydra experimentally deprived of their algae (aposymbiotic hydra) reproduce as well as or better than normal green hydra during starvation. As Park *et al.* (1967) have stated, the variety of experimental conditions and strains of animals used in these various studies renders difficult the interpretation of these contradictory results.

Several previous studies on the effects of light have dealt with the effects of light on the behavior of hydra (Singer, Rushforth and Burnett, 1963; Rushforth, 1966), but little attention has been devoted to the effects of light on growth and reproduction. Also, several previous workers have investigated the effects of population density in hydra, but most of these studies have focused on sexual differentiation (Loomis, 1954; Park, Mecca and Ortmeyer, 1961) and floating behavior (Lomnicki and Slobodkin, 1966). Davis (1966) reported a diffusible substance, possibly protein in nature, released into the medium of crowded *Chlorohydra viridissima* and *Hydra littoralis* which influences budding and regeneration of animals cultured in such media.

Two distinct species of green hydra have been described, *Chlorohydra viridissima* (Pallas, 1766) and *Chlorohydra hadleyi* Forrest, 1959. Nearly all the previous studies on the role of symbiotic algae in hydra have been conducted with hydra usually designated in recent years as *Chlorohydra viridissima*, but often inadequately described. Until Forrest's recent description of *Chlorohydra hadleyi*, all green hydra were generally assigned to a single species usually called either *Chlorohydra viridissima* or *Hydra viridis*.

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As noted previously, certain of the earlier studies have led to some apparently contradictory results. Some of these contradictions may well be due to differences between strains or species of hydra used. The recent work of Park *et al.* (1967) comparing designated strains of the two described species points out a number of distinct morphological and physiological differences between these two green hydras and clearly suggests the possibility of significant differences in the role of algae in the different species. Both Park *et al.*, 1967, and Oschman (1967) provide evidence of morphological differences between the algae of different strains of green hydra.

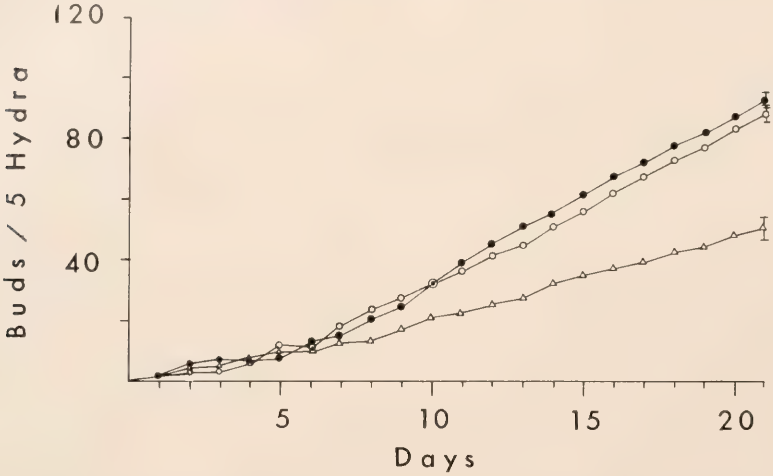

FIGURE 1. Effect of continuous darkness on asexual reproduction of Burnett Green (triangles), Kenilworth Green (closed circles), and Kenilworth Albino hydra (open circles).

The purpose of the present study is to investigate further the interactions between the symbiotic algae and their hosts through an analysis of the effects of light and other factors on asexual reproduction in designated strains of *Chlorohydra viridissima* and *Chlorohydra hadleyi*.

MATERIALS AND METHODS

Two species of green hydra were used in this study: *Chlorohydra viridissima* (Pallas 1766) (Burnett Strain) and *Chlorohydra hadleyi* Forrest, 1959 (Kenilworth Strain). Initial stocks of both strains were obtained from Dr. Helen D. Park of the National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland. Clones were subsequently developed from a single individual of each strain and an aposymbiotic clone was developed from our Kenilworth clone by culturing the green hydra in 0.5% glycerine medium for several weeks (Whitney,

1907) to induce expulsion of the algae. Despite repeated attempts in our laboratory, we were unable to develop a viable culture of aposymbiotic Burnett hydra using glycerine, chloramphenicol, or culture in darkness.

The culture medium (BVS) (modified from Loomis and Lenhoff, 1956) consisted of 100 mg $NaHCO_3$ and 50 mg disodiumethelene-diaminetetraacetate (versene) in a liter of filtered spring water. Stock cultures of several hundred hydra were maintained in three-inch Carolina Culture Dishes containing 50 ml culture medium and kept in foil-lined light tight boxes. Illumination was provided continuously for twelve hours each day (5:00 AM–5:00 PM) by a single fifteen watt fluorescent light (Sylvania Cool-white) placed 10 inches from the top of the culture dishes providing a uniform light intensity of approximately 250 foot candles at the level of the cultures. Temperature was maintained at 20° C ±1°.

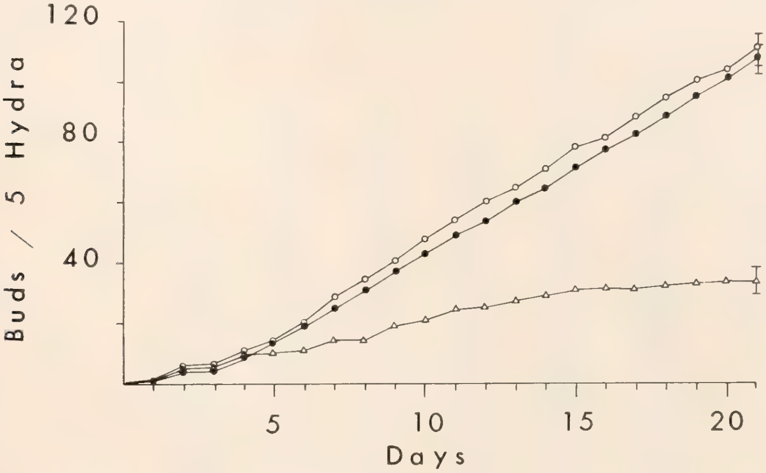

FIGURE 2. Effect of 12 hours light/12 hours darkness on asexual reproduction of Burnett Green (triangles), Kenilworth Green (closed circles), and Kenilworth Albino hydra (open circles).

Cultures were provided with freshly-hatched specimens of *Artemia* nauplii for a period of one hour on alternate days, allowing the hydra to feed to repletion. The cultures were then rinsed and fresh culture medium provided. On non-feeding days the culture solution was changed, and once each week the culture dishes were cleaned to minimize bacterial contamination. These methods provided an environment under which hydra reproduced exclusively by asexual budding during the course of experimentation. Prior to the experiments cultures of all three strains were maintained for several months under these conditions.

Experimental cultures consisted of five uniform hydra selected from stock cultures; uniform hydra defined by Lenhoff and Bovaird (1961) as animals having a single bud in early stages of development and having been starved for one day. Culture conditions for experimental cultures except as specified in particular experiments were as previously stated for stock cultures with two notable exceptions: Culture medium in those maintained under starvation conditions was changed daily, and the period of illumination in the photoperiod experiment was altered manually. Cultures referred to in the results as having been maintained in continuous darkness actually received 10 minutes of ambient laboratory light per day due to the need for light during feeding and counting.

Reproduction was measured each day by counting and removing buds dropped from the five parent hydra in each culture dish.

RESULTS

Effects of light on budding

Three replicate cultures each of Kenilworth Green, Kenilworth Albino, and Burnett Green hydra were cultured under conditions of continuous darkness, 12 hours light/12 hours darkness, and continuous light for a period of 21 days to investigate the effects of light on budding. Light intensities were maintained at 250 foot candles. As illustrated in Figures 1–3, the Kenilworth Green and Albino hydra produced more buds under all three lighting conditions than the Burnett Green hydra ($P < 0.01$). There was no significant difference in the number of buds produced by the Kenilworth Green and Albino hydra under any of the light regimes ($P > 0.05$). Both produced significantly more buds under continuous illumination and under 12 hours of light and 12 hours of darkness than under continuous darkness ($P < 0.01$).

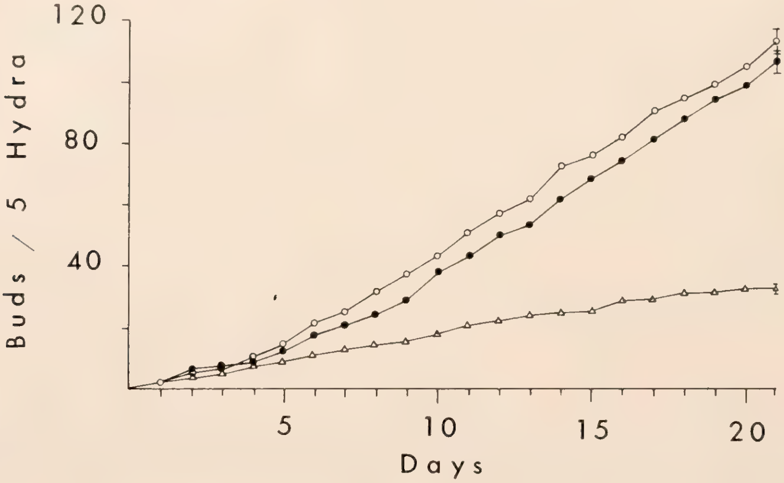

FIGURE 3. Effect of continuous light on asexual reproduction of Burnett Green (triangles), Kenilworth Green (closed circles), and Kenilworth Albino hydra (open circles).

The Burnett Green hydra, in contrast, produced significantly more buds in continuous darkness than under either illumination condition ($P < 0.01$), but there was no significant difference in the number of buds produced under continuous light and 12 hours of light/12 hours of darkness ($P > 0.05$). The data from this experiment, therefore, do not demonstrate any positive relationship between the algae contained in the gastrodermal cells of the hydra and budding in either the Kenilworth Green or Burnett Green hydra. The increased budding observed in the Kenilworth Green hydra under illumination cannot be attributed to the presence of endodermal algae since the Kenilworth Albino hydra exhibited a similar increase. These results are in basic agreement with those of Muscatine (1961), Muscatine and Lenhoff (1965), Stiven (1965), and Park *et al.* (1967), indicating that there was no difference in the budding of green and aposymbiotic hydra under constant illumination when food was not a limiting factor.

Effect of photoperiod on budding

Since the Kenilworth and Burnett Green hydra were differently affected by exposure to light and darkness, another experiment was undertaken to determine the effects of different photoperiods on budding in the two species. Three replicate cultures each of Burnett Green and Kenilworth Albino hydra were grown under continuous darkness and with 2 hours light (11:00 AM–1:00 PM), 4 hours light (11:00 AM–3:00 PM), 8 hours light (5:00 AM–1:00 PM), and 12 hours light (5:00 AM–5:00 PM) per day at an intensity of 250 foot candles for a period of 21 days.

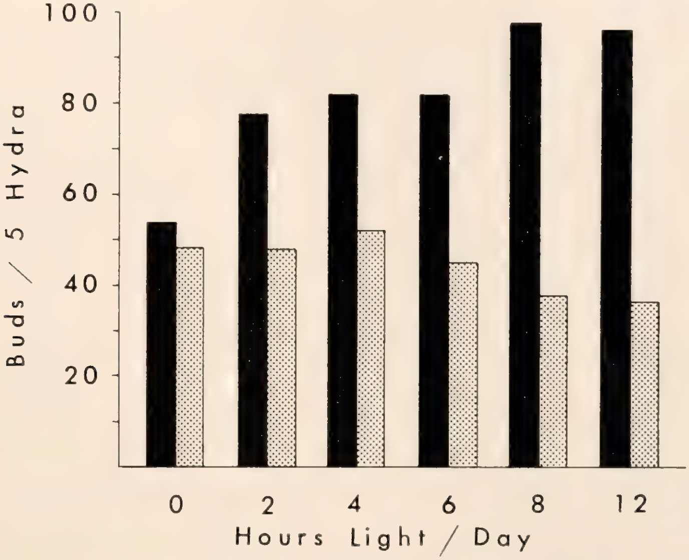

FIGURE 4. Effect of photoperiod on budding in Kenilworth Albino (solid bars) and Burnett Green hydra (stippled bars).

The Burnett Green hydra produced the greatest number of buds from 0–4 hours of light per day and a significantly smaller number of buds from 6–12 hours of light per day (Fig. 4, Table I). The Kenilworth Albino hydra produced the greatest number of buds with the longest photoperiods employed (8 and 12 hours of light) and produced the smallest number of buds under constant darkness (Fig. 4, Table II). No significant difference in budding was observed between

TABLE I

Summary of Duncan's Multiple Range Test comparing budding of Burnett Green hydra under different photoperiods

Hours light	0	2	4	6	8	12
0	—	ns	ns	ns	1	1
2	ns	—	ns	ns	1	1
4	ns	ns	—	5	1	1
6	ns	ns	5	—	1	1
8	1	1	1	1	—	ns
12	1	1	1	1	ns	—

1—$P < 0.01$
5—$P < 0.05$
ns—not significant

TABLE II

Summary of Duncan's Multiple Range Test comparing budding of Kenilworth Albino hydra under different photoperiods

Hours light	0	2	4	6	8	12
0	—	1	1	1	1	1
2	1	—	ns	ns	1	1
4	1	ns	—	ns	1	1
6	1	ns	ns	—	1	1
8	1	1	1	1	—	ns
12	1	1	1	1	ns	—

1—$P < 0.01$
5—$P < 0.05$
ns—not significant

2–6 hours of light with the Kenilworth Albino hydra. Thus the results of this experiment tend to confirm the results of the previous experiment and furthermore, these results demonstrate that budding tends to decrease with increasing exposure to light in the Burnett Green hydra but tends to increase with increasing light in the Kenilworth Albino hydra.

Effects of light intensity on budding

After it was found that Kenilworth Green hydra produced more buds and the Burnett Green hydra produced fewer buds with longer photoperiods, we designed two experiments to investigate the influence of increased light intensity on budding in the two species. In the first experiment three replicate cultures of Burnett Green, Kenilworth Green, and Kenilworth Albino hydra were maintained under constant illumination of 3200 foot candles provided by two 15-watt Sylvania Cool-white fluorescent bulbs located four inches from the top of the culture dishes. The culture dishes were placed in a foil-lined cardboard box to increase the uniformity of illumination and increased ventilation was provided to prevent temperature increase due to the proximity of the lights. Temperature was maintained at 20 ±1° C as in the other experiments.

The results of this increased light intensity indicated a further reduction in the budding of the Burnett Green hydra which produced a total of only eight buds per culture, all of which were produced during the first five days of the 21-day experiment (Fig. 5). The Kenilworth Green and Albino hydra produced 99.3 ± 3.33 hydra per culture and 95.0 ± 3.41 hydra per culture, respectively, during the 21-day period and continued budding at a relatively constant rate throughout the experiment. The difference in budding between the Kenilworth Green and Albino hydra was found not to be statistically significant ($P > 0.05$).

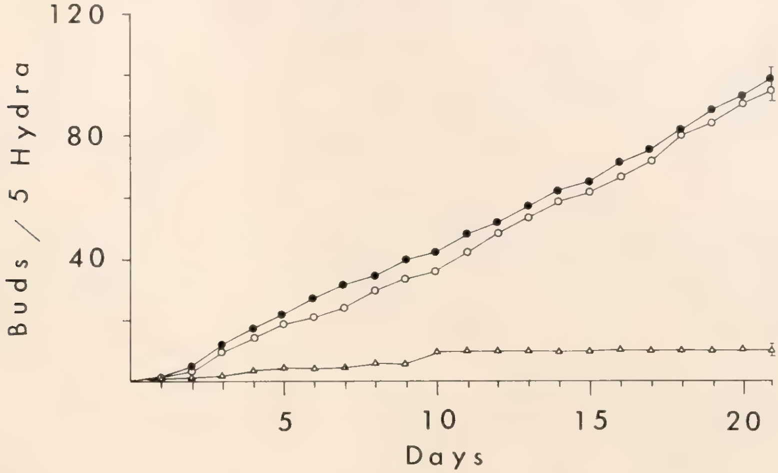

FIGURE 5. Effect of continuous light of 3200 foot candles on asexual reproduction in Burnett Green (triangles), Kenilworth Green (closed circles), and Kenilworth Albino hydra (open circles).

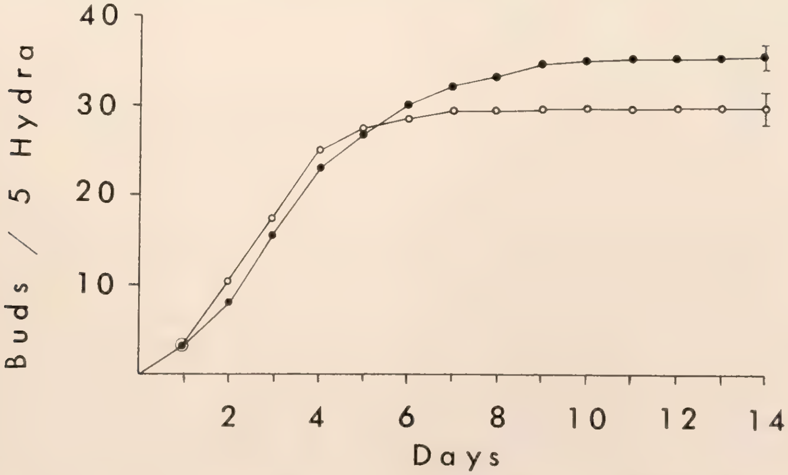

FIGURE 6. Effect of continuous light of 3200 foot candles on asexual reproduction in Kenilworth Green (closed circles) and Kenilworth Albino hydra (open circles) during starvation.

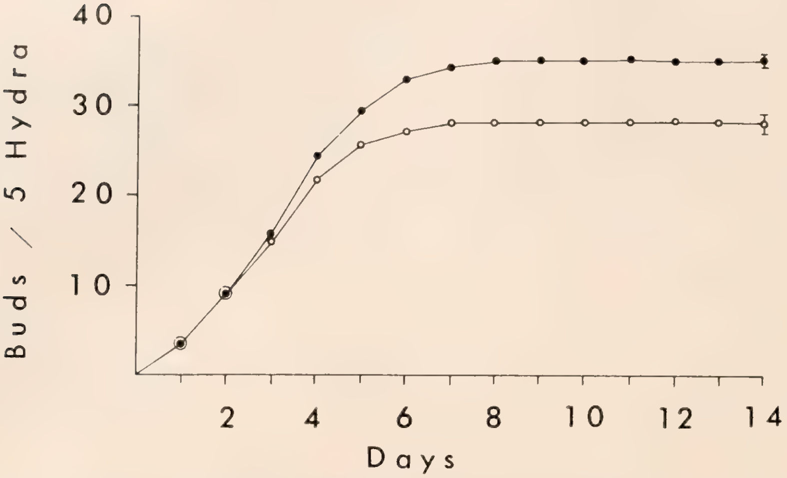

FIGURE 7. Effect of continuous light of 250 foot candles on asexual reproduction in Kenilworth Green (closed circles) and Kenilworth Albino hydra (open circles) during starvation.

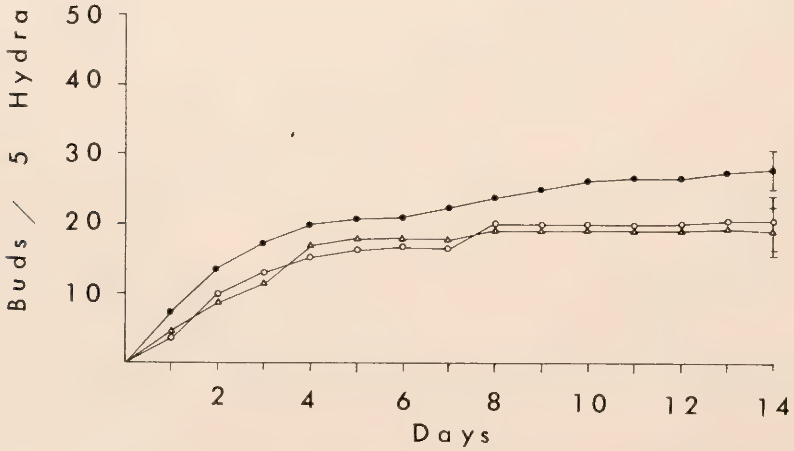

FIGURE 8. Effect of continuous darkness on Burnett Green (triangles), Kenilworth Green (closed circles), and Kenilworth Albino hydra (open circles) during starvation after previous exposure to uncrowded conditions.

Another experiment designed to test the effects of light intensity on budding in green versus albino hydra was conducted with Kenilworth Green and Kenilworth Albino hydra under conditions of starvation for a 14-day period. Three replicate cultures of each strain were cultured under 250 foot candles (Fig. 6) and 3200 foot candles (Fig. 7) of fluorescent illumination. These hydra exhibited a significant difference in the number of buds produced by the green and albino hydra at both intensities ($P < 0.05$), but no significant difference in the number of buds produced by corresponding hydra at the two light intensities ($P > 0.05$).

Therefore these results indicated that under starvation conditions algae make a positive contribution to budding, but that there was no significant difference in the algal contribution in hydra exposed to 250 and 3200 foot candles.

Effect of light and starvation on budding

Since previous results had demonstrated that budding is influenced by both light and starvation, an experiment was conducted to explore the effects of different light regimes under starvation conditions in the three strains of hydra. Three

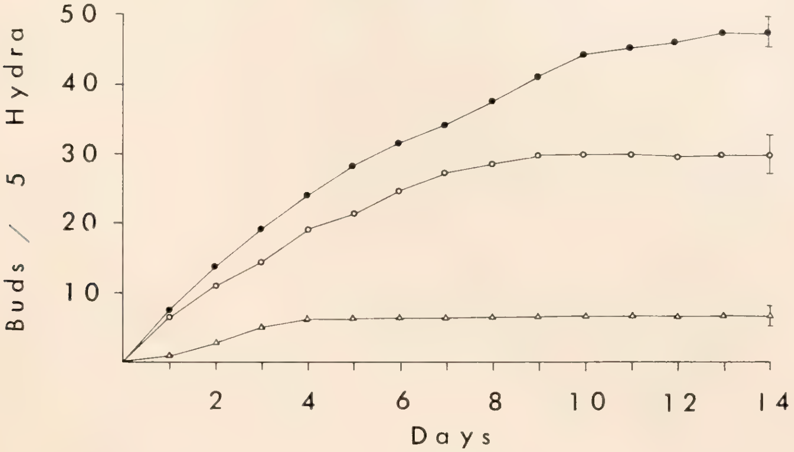

FIGURE 9. Effect of 12 hours light/12 hours darkness on asexual reproduction in Burnett Green (triangles), Kenilworth Green (closed circles), and Kenilworth Albino hydra (open circles) during starvation after previous exposure to uncrowded conditions.

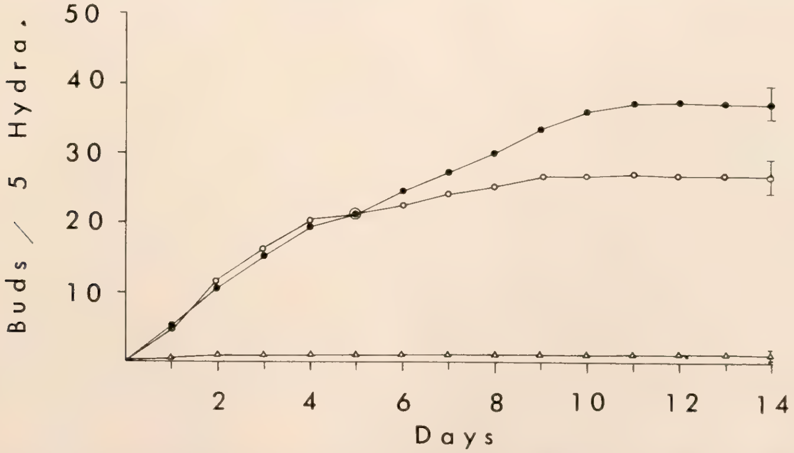

FIGURE 10. Effect of continuous light on asexual reproduction in Burnett Green (closed circles), and Kenilworth Albino hydra (open circles) during starvation after previous exposure to uncrowded conditions.

replicate cultures each of Burnett Green, Kenilworth Green, and Kenilworth Albino hydra were maintained under conditions of continuous darkness, 12 hours of light/12 hours of darkness, and continuous light, and fed on alternate days for 21 days. At the end of this period feeding was discontinued and observations made of the survival and bud production in each of the three strains. Observations were continued for 14 days.

Survival of 100% was observed in all three strains, but significant differences in budding were recorded as shown in Figures 8–10. Under 12 hours light/12 hours darkness the Kenilworth Green and Kenilworth Albino hydra exhibited the greatest budding, producing 47.0 ± 2.20 buds per culture and 29.0 ± 2.99 buds per culture respectively, and continued to produce buds over the longest period of time. Under continuous darkness the inverse was observed. With continuous light the Kenilworth Green and Albino hydra produced fewer buds, but under all three light regimes budding by the Kenilworth Green hydra was significantly higher than that of the Kenilworth Albino hydra (Table III, $P < 0.01$).

Budding in the Burnett Green hydra was greatest in continuous darkness where 19.0 ± 2.69 buds per culture were produced, and was the least under continuous light where only 1.6 ± 0.33 buds per culture were obtained. Under 12 hours

TABLE III

Summary of Duncan's Multiple Range Test comparing budding of three strains of hydra from crowded and uncrowded cultures during starvation and under three different light regimes

		12 hrs light/12 hrs dark						Continuous light						Continuous dark					
		B	G	A	b	g	a	B	G	A	b	g	a	B	G	A	b	g	a
12 hrs light/ 12 hrs dark	B	—	1	1	1	1	5	5	1	1	1	1	1	1	1	1	ns	1	1
	G	1	—	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	A	1	1	—	1	ns	1	1	1	ns	1	ns	1	1	ns	1	1	1	1
	b	1	1	1	—	1	ns	1	1	1	ns	1	ns	ns	1	5	1	ns	ns
	g	1	1	ns	1	—	1	1	1	ns	1	ns	1	1	ns	5	1	1	1
	a	5	1	1	ns	1	—	1	1	1	ns	1	ns	5	1	1	ns	ns	ns
Continuous light	B	5	1	1	1	1	1	—	1	1	1	1	1	1	1	1	1	1	1
	G	1	1	1	1	1	1	1	—	1	1	1	1	1	1	1	1	1	1
	A	1	1	ns	1	ns	1	1	1	—	1	ns	1	1	ns	5	1	1	1
	b	1	1	1	ns	1	ns	1	1	1	—	1	ns	5	1	1	5	ns	ns
	g	1	1	ns	1	ns	1	1	1	ns	1	—	1	1	ns	1	1	1	1
	a	1	1	1	ns	1	ns	1	1	1	ns	1	—	ns	1	5	1	ns	ns
Continuous dark	B	1	1	1	ns	1	5	1	1	1	5	1	ns	—	1	ns	1	ns	ns
	G	1	1	ns	1	ns	1	1	1	1	1	ns	1	1	—	ns	1	1	1
	A	1	1	1	5	5	1	1	1	5	1	1	5	ns	1	—	1	ns	5
	b	ns	1	ns	1	1	ns	1	1	1	5	1	1	1	1	1	—	1	5
	g	1	1	1	ns	1	ns	1	1	1	1	ns	ns	ns	1	ns	1	—	ns
	a	1	1	1	ns	1	ns	1	1	1	ns	1	ns	ns	1	5	5	ns	—

Crowded hydra
B — Burnett Green strain
G — Kenilworth Green strain
A — Kenilworth Albino strain

Uncrowded hydra
b — Burnett Green strain
g — Kenilworth Green strain
a — Kenilworth Albino strain

5 — $P < 0.05$
1 — $P < 0.01$
ns — not significant

light/12 hours darkness the Burnett Green hydra produced 6.6 ± 1.65 buds per culture.

Effects of crowding on budding

Since the animals in the previous experiment had been subjected to different light regimes prior to the period of starvation by nature of the experimental design, a similar experiment was conducted with animals having a common exposure to light during the period immediately prior to starvation. Three replicate cultures of Burnett Green, Kenilworth Green, and Kenilworth Albino hydra were set up using animals taken directly from stock cultures and grown under three different light regimes: constant darkness, 12 hours light/12 hours darkness, and continuous light. Bud production was recorded during a period of 14 days of starvation. These cultures therefore differed in two principal ways from those used in the previous experiment: (1) They had a common history of light exposure for several weeks prior to starvation (12 hours light/12 hours darkness), and (2) they came from stock cultures with a relatively high population density (several hundred hydra per three-inch Carolina Culture Dish) rather than from cultures with a lower population density (five hydra per dish).

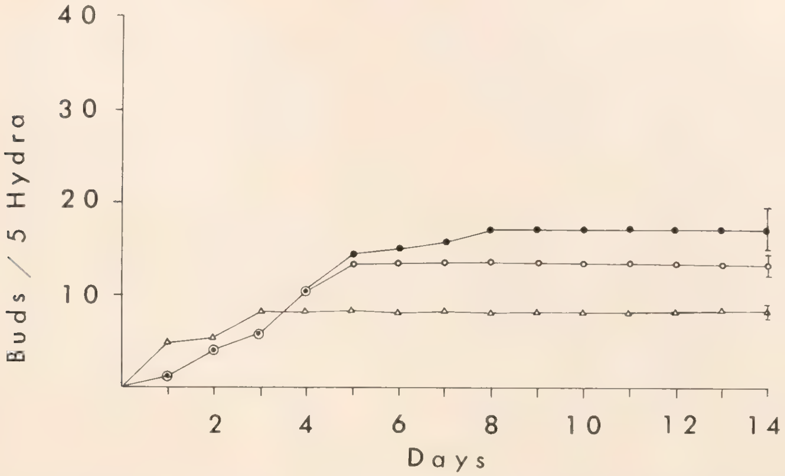

FIGURE 11. Effect of continuous darkness on asexual reproduction in Burnett Green (triangles), Kenilworth Green (closed circles), and Kenilworth Albino hydra (open circles) during starvation after previous exposure to crowded conditions.

The results of this experiment, illustrated in Figures 11–13, indicate that during the 14-day starvation period the Kenilworth Green hydra produced the greatest number of buds in each environment. Budding was significantly higher under continuous light and 12 hours light/12 hours darkness than under continuous darkness (Table III, $P < 0.01$), but no significant difference in budding was observed between constant light and 12 hours light/12 hours darkness ($P > 0.05$). The Kenilworth Albino hydra showed no significant difference in the number of buds produced under any of the three light regimes ($P > 0.05$).

The Burnett Green hydra produced fewest buds under continuous darkness but significantly more buds under both conditions of illumination (Table III, $P < 0.05$). There was no significant difference in budding between the animals grown under continuous light and under 12 hours of light/12 hours darkness ($P > 0.05$). The Burnett Green hydra therefore exhibited a different pattern of budding than that observed in the previous experiments where budding was greatest under constant darkness.

These data indicate that previous exposure to high population densities (crowding) affects the asexual reproduction of green hydra exposed to starvation. Comparing the data from this and the previous starvation experiments, the corresponding Kenilworth Green and Albino hydra produced fewer buds (Table III, $P < 0.05$) subsequent to exposure to crowding conditions than when grown in low density cultures. This decreased budding in hydra exposed to crowding conditions suggests the presence of a lowered metabolic reserve available for budding in these animals. The absence of a significant difference in the budding of Kenilworth Green and Albino hydra starved in continuous darkness indicated the presence of similar levels of metabolic reserves available for budding (Fig. 11). Since the animals were maintained in darkness during the period of experiment, the algae were producing no photosynthetic products which might contribute to budding. In the cultures exposed to light, however, the budding of both types of green hydra, Burnett Green and Kenilworth Green, was significantly greater than under constant darkness (Table III, $P < 0.05$). Furthermore, the budding of the Kenilworth Green hydra was approximately double that of the Kenilworth Albino hydra (Figs. 11–13).

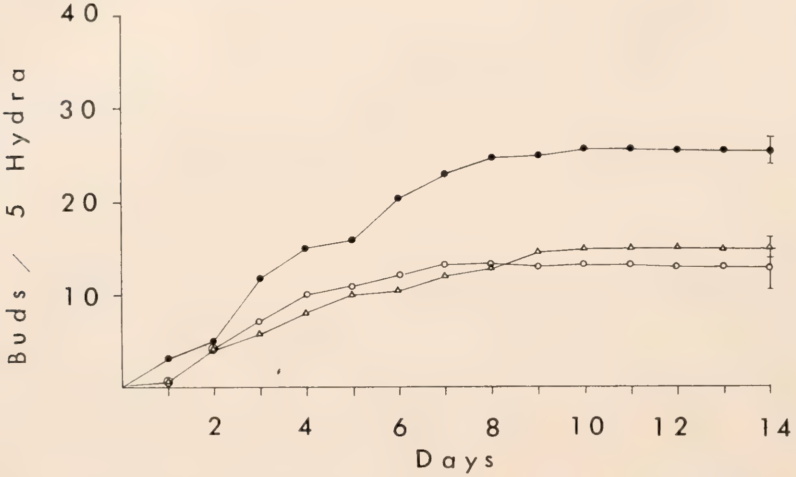

FIGURE 12. Effect of 12 hours light/12 hours darkness on asexual reproduction in Burnett Green (triangles), Kenilworth Green (closed circles), and Kenilworth Albino hydra (open circles) during starvation after previous exposure to crowded conditions.

Exposure to the crowding in the Burnett Green hydra had an effect similar to that on the Kenilworth Green hydra; budding was reduced in animals maintained in the dark as compared to animals under either lighted regime.

Discussion

The results of these experiments demonstrate that light exerts an important influence on budding in both green and albino hydra and that the influence of light is not restricted to its effect on the symbiotic algae of the green hydra. These experiments have shown that budding is the same in both Kenilworth Green and Albino hydra under a wide range of lighting conditions, and that both green and albino hydra exhibit large increases in bud production in lighted cultures (Figs. 1–3). This observation clearly indicates that some factor other than symbiotic algae stimulates budding in the albino hydra and obviously suggests that a similar mechanism may also contribute to the increased budding of green hydra exposed to light.

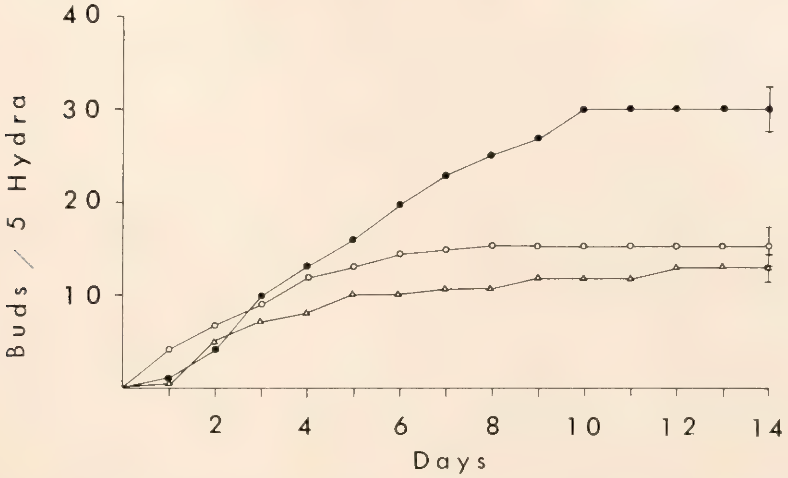

FIGURE 13. Effect of continuous light on asexual reproduction in Burnett Green (triangles), Kenilworth Green (closed circles), and Kenilworth Albino hydra (open circles) during starvation after previous exposure to crowded conditions.

The Burnett Green hydra consistently exhibited lower bud production than the Kenilworth Green or Albino under corresponding conditions, indicating a lower reproductive capacity in this hydra. The most striking difference between the Burnett and Kenilworth hydras is the pronounced difference in the influence of light on budding in the two hydras. Increasing light exposure invariably stimulated budding in the Kenilworth Green and Albino hydras; in contrast, increasing light generally had an inhibitory effect on budding in the Burnett Green hydra.

Well-fed Burnett Green hydra produced fewer buds in constant light and under 12 hours light/12 hours darkness than in constant darkness (Figs. 1–3). Increasing the light intensity from 250 to 3200 foot candles stopped budding in the Burnett Green hydra after about five days while the Kenilworth Green and Albino hydra continued budding at a rapid rate to the end of 21 days (Fig. 5). Photoperiods from 4–12 hours resulted in a significant reduction in budding in the Burnett Green hydra, but the Kenilworth Albino hydra showed increasing bud production with longer photoperiods (Fig. 4).

Experiments with starved animals in lighted cultures demonstrated an increased budding in the Kenilworth Green hydra over that of the Kenilworth Albino hydra in accord with the results of several previous workers, including Goetsch (1924), Muscatine and Lenhoff (1965), and Stiven (1965). These previous investigators have all interpreted the greater budding of the green animals as evidence for a contribution of the symbiotic algae to the nutrition of the host; data from the present experiments show that light may also stimulate budding in starved albino hydra (Figs. 8–10). Albino hydra previously exposed to crowded culture conditions, however, showed no significant difference in budding rates with varied illumination (Figs. 11–13).

Crowding also was found to affect the response of Burnett Green hydra to light. Light exposure inhibited budding in starved Burnett Green hydra from uncrowded cultures but resulted in a stimulation of budding in animals from crowded cultures. Davis (1966) demonstrated the accumulation of a diffusible substance, possibly protein in nature, in the medium of crowded *Chlorohydra viridissima* and *Hydra littoralis*. This substance was found to decrease both the rate of budding and the rate and extent of regeneration of experimental animals.

Evidence from the present experiments suggests that budding may be regulated by the available supply of some critical metabolite(s) in the hydra tissues. The hypothesized metabolite appears to be derived both from feeding and from the photosynthetic activity of the symbiotic algae since well-fed Kenilworth Green and Albino hydra show no difference in reproduction (Figs. 1–3) and since light stimulates budding in starved Kenilworth Green hydra (Figs. 8–10). The decreased budding of starved Kenilworth Green and Albino hydra exposed to crowding as compared to Kenilworth Green and Albino hydra from uncrowded cultures (Table III) further suggests that crowding affects the pool size of this hypothesized metabolite, possibly via the diffusable inhibitor demonstrated by Davis (1966) in the medium of crowded cultures. Starved Kenilworth Green animals, with smaller pools of the metabolite, demonstrate the release of the metabolite by the algae through their increased budding in lighted cultures (Figs. 11–13).

The Burnett Green hydra appear to maintain lower pool sizes of the hypothesized metabolite(s) than Kenilworth Green hydra exposed to similar environmental conditions, as reflected in their lower bud production. Furthermore, Burnett Green hydra appear to be very susceptible to increased levels of the metabolite since most of the experiments showed an inhibitory effect of light on budding in this hydra; perhaps in such experiments the combined contribution of the metabolite from feeding and from algal photosynthesis resulted in an effect resembling substrate inhibition.

Burnett Green hydra from crowded cultures, like the Kenilworth Green hydra from crowded cultures, showed evidence of a lowered pool size of the metabolite.

In the case of the Burnett Green hydra, however, this decreased pool size resulted in an apparent reversal in the effect of light on budding of the crowded animals. Thus we postulate that the mechanism of budding and the role of the algae in green hydra in the two strains of green hydra as revealed in these experiments is explainable on a common basis, assuming only quantitative differences in pool sizes and possibly differences in inhibitory levels of the critical metabolites.

Our experiments were not designed to provide evidence regarding the chemical nature of the critical metabolites. The work of Muscatine (1965) and Muscatine *et al.* (1967), however, on algae isolated from other strains of green hydra (Florida Strain 1961, Carolina Strain 1960, and Lenhoff's mutant strain) has demonstrated that these algae liberate significant amounts of maltose and/or glucose into the incubating medium. These results therefore offer one suggestion as to the possible nature of the hypothesized metabolites responsible for the observed differences in budding in our experiments.

We wish to thank Mr. Robert F. Browning and Dr. Helen D. Park for their helpful suggestions, Mr. George T. Barthalmus for his advice and assistance in the development of clones of experimental animals, and Mr. Joel Katz for assistance in data processing.

Summary

1. Experiments were conducted with three strains of green and aposymbiotic hydra to study the effects of light on asexual reproduction. Animals used were the Kenilworth Green and Kenilworth Albino (aposymbiotic) strains of *Chlorohydra hadleyi* and the Burnett Green strain of *Chlorohydra viridissima.*

2. Budding of both the Kenilworth Green hydra and the Kenilworth Albino hydra was significantly greater in constant light and with 12 hours light/12 hours darkness than under constant darkness, indicating that some factor other than the green algae promotes budding of albino hydra in lighted cultures.

3. Budding was less in Burnett Green hydra in constant light and 12 hours light/12 hours darkness than under continuous darkness.

4. Budding increased with increasing photoperiods in the Kenilworth Albino hydra but decreased with increasing photoperiods in the Burnett Green hydra.

5. No difference was observed in the budding of Kenilworth Green and Albino hydra at 250 and 3200 foot candles, but budding was greatly reduced in Burnett Green hydra at 3200 foot candles.

6. Kenilworth Green hydra produced more buds during starvation when exposed to constant darkness, 12 hours light/12 hours darkness, and constant light than Kenilworth Albino hydra.

7. Prior exposure to crowding conditions resulted in decreased budding in starved Kenilworth Green and Albino hydra grown in constant darkness, 12 hours light/12 hours darkness, and constant light. Similarly treated Burnett Green hydra, however, showed increased budding under all three light regimes.

8. The results suggest that budding in green and albino hydra is regulated by levels of some undefined critical metabolite which can be derived both from food

and symbiotic algae. Burnett Green hydra appear to maintain smaller metabolic pools of this substance than Kenilworth Green and Albino hydra, and excessive levels of this metabolite tend to inhibit budding in this strain.

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HABITUATION OF THE WITHDRAWAL RESPONSE IN NEREID POLYCHAETES

1. THE HABITUATION PROCESS IN *NEREIS DIVERSICOLOR*

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The withdrawal response of many polychaetes is easily observable and is elicited by a variety of different stimuli so that it is admirably suited to studies of the habituation process. A rapid withdrawal response is mediated through giant axons in the ventral nerve cord. Five of them have been described in *Nereis virens* by Bullock (1945) and in *N. diversicolor* by Smith (1957) and separate functions have been ascribed to them in *N. virens* by Bullock (1948): the single median fiber normally conducts impulses from the afferent neurones in the anterior region, the paired paramedials conduct from afferents in the posterior region and the paired lateral fibers are excited by stronger stimulation at any point. Each fiber transmits in both directions from the point of excitation.

In studies of habituation of the withdrawal response, sabellids and serpulids have received most attention (*e.g.*, Bohn, 1902; Hargitt, 1906; 1909; Yerkes, 1906; Hess, 1914; Rullier, 1948; Nicol, 1950; Krasne, 1965), but habituation has also been studied in the nereid, *N. pelagica*, by Clark (1960ab). Withdrawal is elicited by several different stimuli in these worms but, in nearly all cases, the response habituates to repeated stimulation although the rate varies according to the nature of the stimulus. Despite the attention habituation of the withdrawal response has received in these worms, little is known about the habituation process itself. Several authors have treated the withdrawal response as an "all or nothing" reflex, and although this may be so in some polychaetes, there is evidence that in *Mercierella enigmatica* (Rullier, 1948) and *Branchiomma vesiculosum* (Krasne, 1965) it is not identical at each stimulus presentation, and that habituation may involve the gradual loss of a response rather than the sudden loss of an "all or nothing" response. From preliminary observations of habituation in *Nereis diversicolor* it was clear that habituation was a complex process in this polychaete and, consequently, it was decided to record successive responses to stimuli by photography so that changes which might occur during habituation, such as in extent of response, rate of contraction and stimulus-response latency could be measured.

Observations on the mud flats populated by *N. diversicolor* showed that habituation is a normal feature of the worm's behavior. Initially individuals feeding on the surface mud are particularly sensitive to sudden stimulation, and great care is needed if an observer is to get sufficiently close to the worms to observe their feeding habits, without eliciting the withdrawal response. Extended worms respond to mechanical disturbances caused by objects dropped onto the mud, tactile stimuli and shadows. Controlled field experiments are not possible, but habituation can be demonstrated to repeated mechanical disturbances and shadows. Initially the worms almost invariably respond to these stimuli by withdrawing into their burrows, but they re-emerge, usually after several minutes delay, and eventually come to ignore the stimulus.

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In the laboratory individuals react to stimuli that are effective in the field and habituate to repeated presentations of them. Observations supported Clark's (1960a) view that withdrawal responses to sudden increases in illumination are different from those to sudden decreases in illumination, moving shadows and mechanical stimuli; they are slower and have longer stimulus-response latencies. Consequently the habituation process has been investigated in *N. diversicolor* by photographing successive responses to sudden decreases in illumination, because it is believed that responses to this stimulus are characteristic of those to a number of other stimuli, and to sudden increases in illumination, which elicit different responses.

METHOD

Sixteen specimens of *N. diversicolor* were subjected to consecutive sudden decreases in light intensity, from 30 ft-c to 5 ft-c, at 30 second intervals. In each trial the light was off for two seconds. Responses were photographed using a Paillard-Bolex H. 16 Reflex camera at 32 frames per second. Developed film was examined frame by frame in a film editor; the stimulus-response time latency, time taken to complete contraction, amount of contraction, length of the worm at the onset of each trial and irrigating behavior were recorded for each individual.

General background illumination was provided by reflected light from a 60 watt, white, tungsten-filament bulb in a bench lamp, directed at one of the walls of the dark room in which the experiment was performed. More intense light was provided by a similar bench lamp arranged above the worms; this lamp was switched off at each trial so that there was a sudden decrease in illumination. Light intensities were measured by an S. E. I. exposure photometer using a standard white magnesium carbonate block.

Worms were kept in 70% sea water in a constant temperature room at 16° C for ten days before the experiment. They were provided with 10 cm lengths of glass tubing; only worms inhabiting tubes were selected for experimentation. Within these tubes nereids carry out apparently normal tubiculous behavior (see *e.g.*, Wells and Dales, 1951). In order to facilitate measurement of contractions the tubes containing *Nereis* were placed on millimeter graph paper in an enamelled dish during the experiment. The dish contained 70% sea water and was partially immersed in a constant temperature bath so that the temperature could be maintained at 16° C. Worms were light adapted under the experimental conditions for one hour prior to the first stimulus.

Sixteen specimens of *N. diversicolor* were also subjected to forty sudden increases in illumination from 5 ft-c to 30 ft-c. Apart from the nature of the stimulus the experimental procedure was identical to that described above.

TABLE I

Stimulus-response latencies and rates of contraction to sudden changes in illumination

Stimulus:	Sudden decrease in light intensity			Sudden increase in light intensity		
Trials	Nos. observations	Mean rate contraction (mm/sec)	Mean latency (sec)	Nos. observations	Mean rate contraction (mm/sec)	Mean latency (sec)
1	11	17.3	0.42	7	6.5	0.71
1–10	29	12.5	0.43	51	4.7	0.73
11–20	11	9.5	0.45	38	4.2	0.61
21–30	13	8.9	0.42	24	4.2	0.63
31–40	7	10.5	0.38	32	4.5	0.69

RESULTS

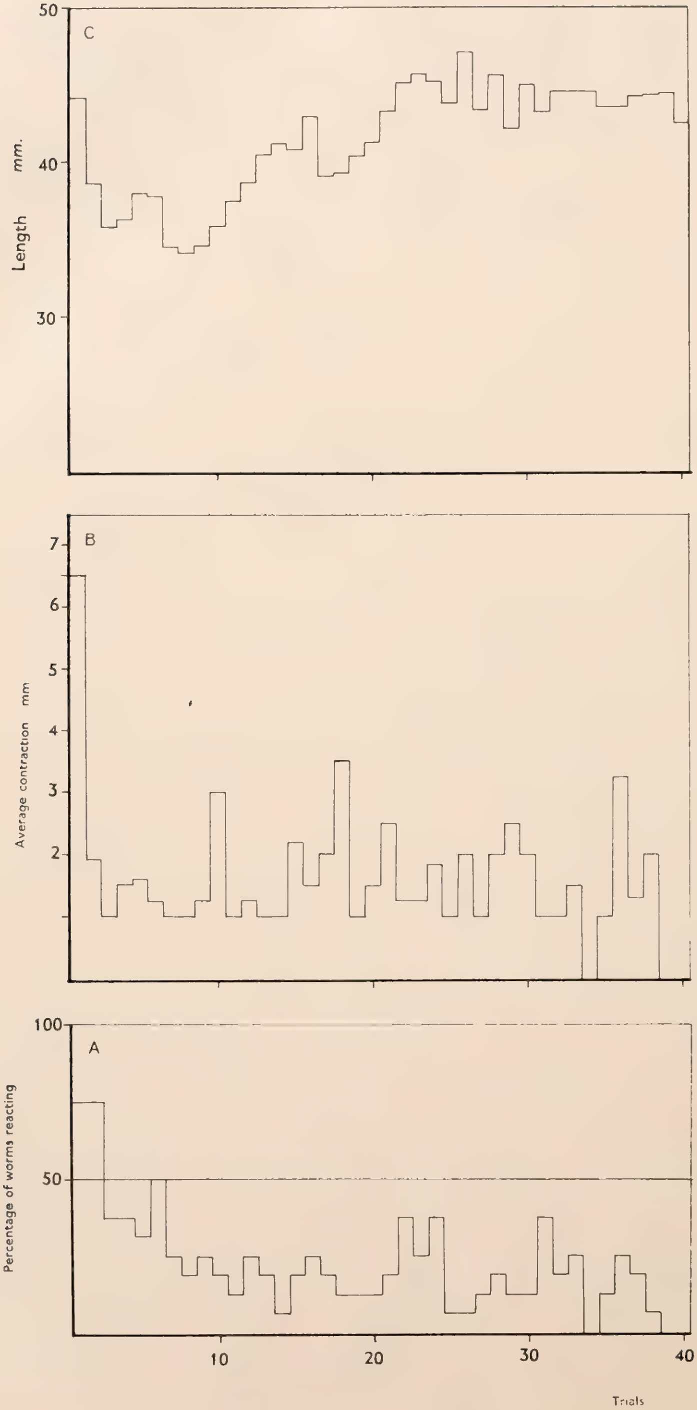
It is clear from the analysis of the photographs that habituation to changes in illumination is a complex process. Habituation to sudden decreases in light intensity can be considered from several aspects:

1. The number of worms responding diminishes with successive trials. Initially 75% of individuals responded to sudden decreases in light intensity, but the number reacting gradually decreased until at the completion of the experiment no worms were responding (Fig. 1A), although it is unlikely that habituation was complete even at this stage.

2. The contraction to the first stimulus was much more extensive than to other stimuli, but subsequently there was no decline in the average extent of contraction of worms (Fig. 1B). In fact, the mean contraction from trials 2–14 (1.44 mm) was actually less than that during the rest of the experiment (1.74 mm). However, any tendency for the extent of contraction to decrease during the first part of the experiment may have been obscured because contractions were limited by incomplete recovery during the intertrial period. Worms which contracted did not extend to their original lengths, and initially there was a cumulative contraction to stimuli (Fig. 1C). Responses were limited during this period, because at each trial the worms were already in a state of at least partial contraction. Subsequently recovery was more rapid, and worms extended to their original lengths during the intertrial period so that maximal contractions were possible.

3. The stimulus-response latencies of contractions of 2 mm or more in length were approximately constant during the experiment and, after the first response, which was the most rapid, the rate of contraction did not apparently diminish (Table I). Responses of less than 2 mm have not been measured because of the inaccuracies of assessing their start and completion.

4. Stimulation not only elicited the withdrawal response in *N. diversicolor* but also inhibited irrigating movements. The responses were partially independent, because although they both involve the longitudinal muscles of the body wall so that a complete contraction necessarily inhibits irrigating activity, incomplete contractions frequently did not inhibit irrigations, and conversely sometimes irrigating movements stopped when there was no contraction. Habituation of the two responses was also independent, and the inhibition of irrigating movements


C
Length mm.
50
40
30
B
Average contraction mm
7
6
5
4
3
2
A
Percentage of worms reacting
100
50
10
20
30
40
Trials

habituated extremely rapidly. The first three stimulations inhibited irrigation in most of the worms but thereafter inhibition was infrequent (Fig. 2A).

Responses to sudden increases in illumination differed from those to sudden decreases in several ways:

1. Responses to sudden increases were slower and had longer stimulus-response latencies than those to sudden decreases (Table I); the means for both rates of contraction and stimulus-response latencies, calculated from the mean values for each individual worm ($n = 16$), were significantly different in both cases ($P < 0.01$; "t" test).

2. 69.7% of responses were posterior end withdrawals whereas nearly all contractions to sudden decreases in illumination were withdrawals of the anterior end (95.8%).

3. Sudden increases in illumination inhibited irrigation in only 1.3% of trials whereas sudden decreases inhibited it in 16.1% of trials. These differences can probably be related to the fact that irrigating movements are normally confined to the anterior half of the animal, which is usually involved in responses to sudden decreases but not sudden increases in illumination.

In many ways the habituation process to sudden increases in illumination is similar to that already described for sudden decreases, but there are several characteristic differences:

1. The initial sudden decline in numbers responding, which occurred to sudden decreases in illumination, did not occur to this stimulus (Fig. 2B; *cf.* Fig. 1A). Fewer worms responded to the first two sudden increases than sudden decreases, but there were subsequently more responses to the former stimulus, and habituation to it was clearly incomplete at the end of the experiment.

2. For the first six sudden increases in illumination, responses diminished in extent in an irregular manner which contrasts with the sudden loss of the initial extensive contraction to sudden decreases in illumination (Fig. 2C; *cf.* Fig. 1B). Thereafter there was no apparent decline in the extent of contractions to either stimulus. Initially contractions to sudden increases in illumination were cumulative, and recovery was not completed in the intertrial period (Fig. 3A).

3. Apart from the first trial, contractions to sudden increases in illumination were generally more extensive than those to sudden decreases (*cf.* Figs. 1B and 2C).

In other respects the habituation process to sudden increases in illumination was similar to that to sudden decreases.

1. The stimulus-response latencies of responses to sudden increases were longer than those to sudden decreases throughout the experiment (Table I) and, apart from trial 11–20, remained approximately constant.

2. Like contractions to sudden decreases, the first sudden increase in illumination elicited the fastest contraction, although thereafter there was no apparent decline in the rate of contracting (Table I).

Individuals often habituate to either sudden increases or sudden decreases in light intensity in an irregular manner, and sometimes it is not clear from an

FIGURE 1. A, Percentage number of worms responding to a series of sudden decreases in illumination; B, Mean contractions of worms responding to a series of sudden decreases in illumination; C, Mean lengths of worms, subjected to a series of sudden decreases in illumination, measured at the onset of each trial.

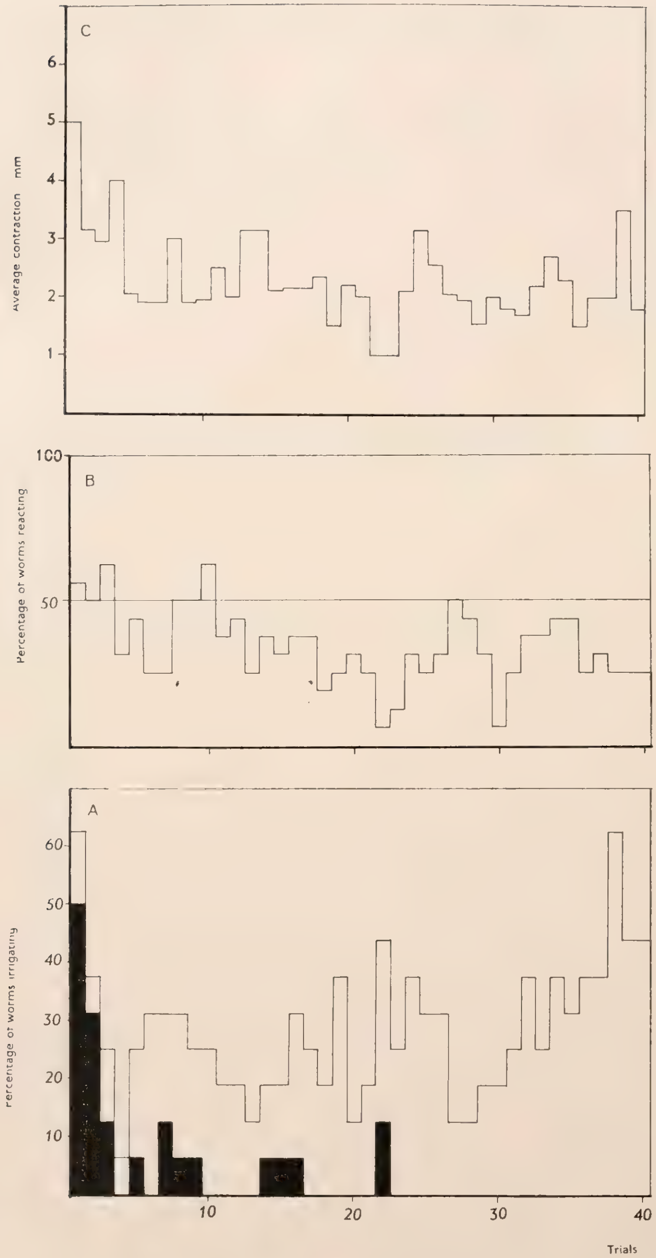


FIGURE 2. A, Percentage numbers of worms irrigating at each trial (empty blocks) and the percentage that stop irrigating when stimulated by sudden decreases in illumination (filled blocks); B, Percentage numbers of worms responding to a series of sudden increases in illumination; C, Mean contractions of worms responding to sudden increases in illumination.

individual performance that habituation is occurring at all (Fig. 3B) and, even in more typical performances, apparently habituated worms may suddenly react to the stimulus (Fig. 3C). Several factors may contribute to this irregularity; the influence of the length of the worms at the time of stimulation has already been discussed, and from observations it seems that worms extended from the ends of their tubes are particularly sensitive to stimulation, whereas individuals crawling along their tubes or reversing in them are relatively insensitive.

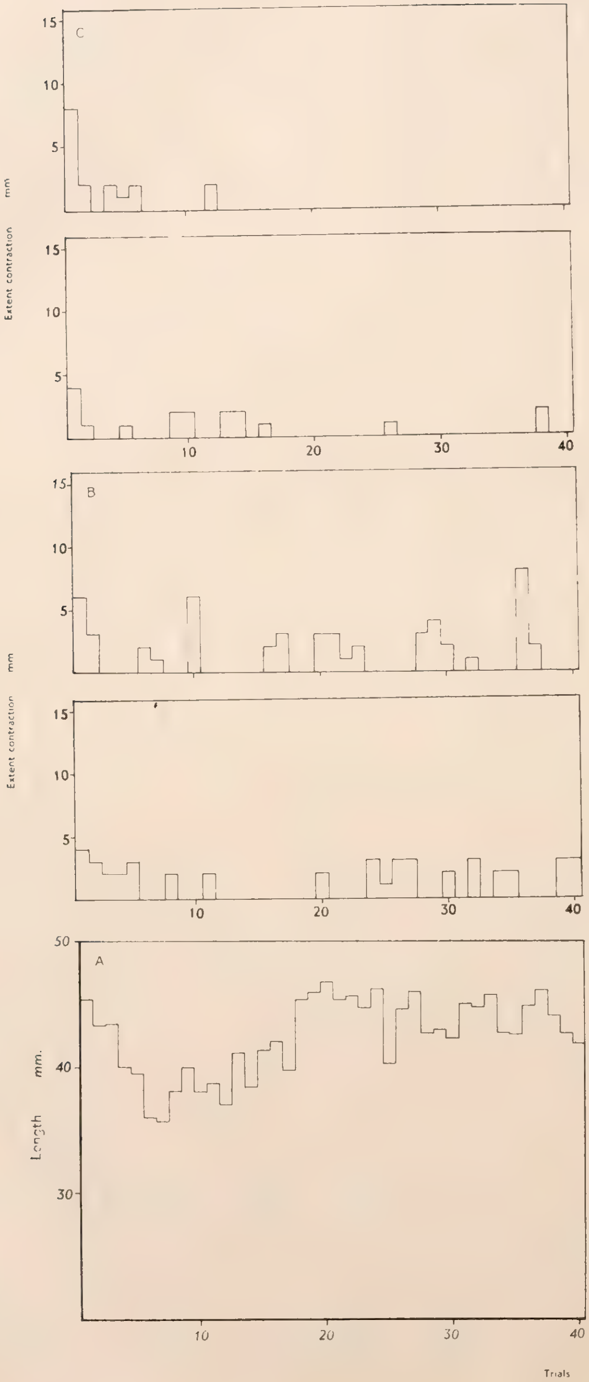
Discussion

Habituation in *Nereis* is probably a complex, central process which cannot be solely attributed to either sensory or motor accomodation. Accomodation of synapses may be an integral part of the process, and it has already been shown that sensory-giant axon synapses and motor-giant axon synapses accommodate to repeated electrical and tactile stimulation in *N. virens* (Horridge, 1950) and to sudden changes in illumination in *N. virens*, *N. diversicolor* and *Platynereis dumerilii* (Gwilliam, 1969). However, sensory adaptation is precluded from being entirely responsible for habituation, because the withdrawal response may be replaced during the habituation process by alternative behavior. Withdrawal to a posterior tactile stimulus is replaced by the posterior end bending away from the source of stimulation in *N. virens* (Horridge, 1950), and withdrawals to anterior tactile stimuli are often replaced by worms (*N. pelagica*, *N. diversicolor*, and *P. dumerilii*,) attacking the seeker used to stimulate them (Evans, 1969).

The withdrawal response itself changes in nature during habituation, and it is likely that one response is replaced by another before there is complete failure to respond. The initial extensive contractions are almost certainly giant axon responses, except possibly the responses to sudden increases in illumination which are slower, but the incomplete contractions that replace them are less likely to be mediated through the giant axons. Indeed, Gwilliam (1969) has made electrophysiological recordings from the ventral nerve cords of nereids and has found that giant axon potentials may occur to the first few stimulus presentations (light or mechanical shocks), but when these disappear bursts of fine fiber activity can still be recorded from the cord when the animal is stimulated.

Worms which have habituated to one stimulus are still capable of responding if the nature of the stimulus is changed (Clark, 1960a) so that neuro-muscular synaptic accommodation has also been excluded as an explanation of habituation. However, accommodation of this junction could act as a safety device if there was a continuous barrage of different stimuli which might otherwise lead to overstimulation of the withdrawal response.

There is evidence that habituation is a central, rather than peripheral, process in other invertebrates. Dethier (1952) found that habituation of proboscis eversal and drinking elicited by tarsal stimulation with a sucrose solution, in the blowfly *Phormia regina* could "spread" from one leg to the contralateral leg. A similar "spread" of habituation has been observed in the earthworm by Kuenzer (1958). Habituation to a tactile stimulus on one part of a segment extends to other parts of the segment and to neighboring segments in diminishing strength as the distance from the original stimulation increases.


C
15
10
5
Extent contraction mm
15
10
5
10
20
30
40
B
15
10
5
Extent contraction mm
15
10
5
10
20
30
40
A
50
40
30
Length mm.
10
20
30
40
Trials

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SUMMARY

1. Habituation of the withdrawal response in *Nereis diversicolor* has been investigated by photographing successive responses to sudden decreases and sudden increases in illumination.

2. Responses to sudden decreases in illumination are usually anterior end withdrawals whereas sudden increases usually elicit posterior end withdrawals. Withdrawals to sudden decreases are faster and have shorter latencies than those to sudden increases.

3. Most worms responded to the first stimulation in a series by withdrawing, but the number responding diminished with successive trials. Although there was an initial decline in the extent of withdrawals, subsequent contractions were approximately constant.

4. Sudden decreases in illumination sometimes inhibit irrigating movements, particularly when the stimulus is novel. However, habituation is rapid and apparently independent of habituation of the withdrawal response; after the third trial irrigating activity was infrequently inhibited by stimulation although withdrawals continued to occur.

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Figure 3. A, Mean lengths of worms, subjected to sudden increases in illumination, measured at the onset of each trial; B, Erratic responses of individual worms, upper figure: to sudden decreases in illumination, lower figure: to sudden increases in illumination; C, Typical habituation of individual worms, upper figure: to sudden decreases in illumination, lower figure: to sudden increases in illumination.

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HABITUATION OF THE WITHDRAWAL RESPONSE IN NEREID POLYCHAETES

2. RATES OF HABITUATION IN INTACT AND DECEREBRATE WORMS

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Most nereid polychaetes are tubiculous or burrowing worms which come to the ends of their tubes or burrows in order to feed. When exposed in this way the worms are highly susceptible to attack from a wide variety of predators including fishes (*e.g.*, see Scott, 1901, 1902; Jones, 1954; Rae, 1956) birds (Witherby, Jourdain, Ticehurst and Tucker, 1938–41), and crustaceans (Blegvad, 1914). It is not surprising, therefore, that the worms have well developed withdrawal responses that are elicited by almost any sudden stimulus.

It is now well established that the worms habituate to repeated, innocuous stimulation (Clark, 1960ab; Evans, 1969). Habituation of escape responses is usually considered to allow for a compromise between the conflicting needs of the animal: the need to respond and escape from its enemies, on the one hand, and the need to continue with normal activities, such as feeding, on the other (*e.g.*, Nicol, 1950). Habituation of the withdrawal response almost certainly subserves this function in nereids, because under natural conditions the worms are undoubtedly subjected to stimulation from both innocuous and potentially harmful agents; shadows, for example, may be caused by floating seaweed or by passing clouds but also by predators.

The withdrawal response habituates at different rates according to the nature of the stimulus, and it has been suggested that the rate of habituation is related to the significance of the stimulus in terms of the likelihood of it heralding the approach of a predator (*e.g.*, Clark, 1960b). It is believed that there is a hierarchy of stimuli of increasing urgency to which the worms habituate less and less rapidly. Tactile stimuli, for example, are highly significant, because they indicate contact between the predator and its prey, and the serpulid *Hydroides dianthus* habituates more slowly to these stimuli than to shadows, which are less urgent (Yerkes, 1906). Similarly, the sabellid *Branchioma vesiculosum* habituates to shadows but not at all to tactile stimuli (Nicol, 1950) unless the intensity of the stimulus is controlled, in which case habituation is slow (Krasne, 1965).

The normal background of stimulation experienced by worms also influences their reactivity to stimuli. Worms either do not react at all, or have poorly developed responses to stimuli which they do not usually experience. Hargitt (1909) found that specimens of *Hydroides* collected from shallow water, where shadows are normally experienced by the worms, responded to shadows, whereas worms collected from deep water, where the light is perpetually dim and shadows are not normally experienced, did not react to them.

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In the present account, habituation of the withdrawal response has been compared in three nereids to a variety of stimuli. These worms have been collected from Millport, Scotland, where they are found in entirely different habitats and therefore provide an excellent opportunity for comparing the influence of the normal environmental experience of the worms on their rates of habituation to different stimuli.

The nereids chosen for this study, *N. diversicolor, N. pelagica* and *Platynereis dumerilii*, are all common at Millport. *N. diversicolor* lives in burrows which are basically U-shaped but in some circumstances may consist of ramifying galleries (Dales, 1950; Bogucki, 1954; Harley, 1956). At Millport it has been collected from Kames Bay, which is sheltered but subjected to more wave action than the estuarine mud flats, which are the characteristic habitats of this species. There is a lack of brown algae or other agents likely to cause innocuous shadows at Kames Bay.

N. pelagica is found on rocky shores where there are dense growths of seaweeds such as *Fucus* and *Laminaria*, where it is likely to be subjected to a good deal of innocuous stimulation from shadows and mechanical disturbances. At Millport it has been collected from East Flats and Keppel Pier, which are both typical habitats; it is common under stones and in *Laminaria* holdfasts. *N. pelagica* is usually considered to be the typically "errant" nereid, but I have frequently found individuals in poorly constructed mucus tubes under stones.

P. dumerilii is found in grey mucus tubes attached to decaying seaweed and other solid objects at a depth of about 20 m off Kames Bay, Millport (Clark and Milne, 1955), where it has been collected for the present work. At this depth in the sea there is a considerable reduction in light intensity (Atkins, 1945) so that this species might be expected to react to light stimuli differently from *N. diversicolor* and *N. pelagica.*

The supra-oesophageal ganglion is important in the more elaborate behavioral modifications of which nereids are capable (Evans, 1963ab), but its importance in habituation has never been investigated. As a complementary study, this has been investigated by comparing rates of habituation in intact and decerebrate worms.

METHOD

P. dumerilii, N. diversicolor and *N. pelagica* have been collected at Millport, Scotland and the experiments carried out in the laboratories of the Scottish Marine Biological Association at Millport.

The experimental procedure is basically the same as that described in the first part of this paper (Evans, 1969). It differs only in the manner of recording responses and other minor details.

In the present investigation, responses to stimuli were observed for 30 trials at half minute intervals and recorded in terms of the extent of contraction to each stimulus; two or three individuals were observed simultaneously. After collecting, worms were provided with glass tubes, which they inhabited, and kept in tanks circulating with fresh sea water for 3–14 days. During the experiment

TABLE I

Number of worms used in each experiment

Stimulus		Sudden changes in illumination			Mechanical shocks	Sudden decreases and Mechanical shocks	Anterior tactile	Posterior tactile
Species		Sudden increase	Sudden decrease	Shadows				
		Worms in tubes						
N. diversicolor	intact	11	12	10	22	12	11	12
	decerebrate	10	10	10	12	9	10	9
P. dumerillii	intact	18	11	8	16	11	10	10
	decerebrate	20	24	23	10	11	10	10
N. pelagica	intact	10	10	8	11	13	10	10
	decerebrate	10	10	9	10	10	7	10
		Worms not in tubes						
N. diversicolor	intact	10	10	10	10		10	10
	decerebrate	10	10	10	10		10	10
P. dumerilii	intact	10	10	10	18		10	11
	decerebrate	10	10	10	15		10	10
N. pelagica	intact	13	10	10	13		10	10
	decerebrate	10	10	10	13		10	10

they were maintained at the temperature of the sea water tanks by immersing the dish containing them in a temperature conrolled water bath.

Six different stimuli were used: mechanical shocks, tactile stimuli to the anterior and to the posterior ends, sudden increases in illumination (2 ft-c to 30 ft-c), sudden decreases in illumination (30 ft-c to 2 ft-c) and shadows. Shadows were achieved by passing a piece of hardboard, 24″ × 12″, between the more intense of the two light sources and the worms, in about two seconds, so that it caused a decrease in light intensity from 30 ft-c to about 2 ft-c; tactile stimuli by a blunt seeker; and mechanical shocks by a pendulum, consising of a 200 g brass weight suspended by 20 cm of thread, striking the side of the dish after being released from a distance of about 10 cm from it. At least 7 worms were subjected to each stimulus (Table I).

In the first series of experiments worms remained in the glass tubes they were inhabiting throughout the experiment. But in a second series worms were evicted from their tubes and then tested to a series of stimulations while they were crawling or swimming in the dish containing them.

Decerebration was performed in the manner described by Clark and Evans (1961) except that it was found necessary to cauterize the wounds. MS222 (Sandoz Products) was used as an anesthetic; recovery from this is extremely rapid, but worms were not used until the day after the operation.

Theoretically, there are several ways in which animals can habituate. An animal may, for example, be highly reactive initially but habituate rapidly to the stimulus; alternatively, it may be relatively unreactive at first but then habituate slowly. Clearly, a consideration of both the habituation rate and the frequency of responding gives a much clearer indication of the animal's behavior towards a series of stimulations than either of these factors alone and, consequently, in the present work, the habituation rates and response frequencies have been assessed for each worm to each stimulus.

The response frequency has been measured for each individual by calculating the mean number of responses that occurred during the 30 trials given in each experiment. Mean performances to different stimuli have been compared statistically by using a "t" test. The mean contractions have also been calculated to each stimulus.

It is unsatisfactory to measure the habituation rate by using a criterion of habituation of, say five or ten responseless trials, because responses occur after a period of unreactivity in some worms but not in others (Evans, 1969). In order to take into account both the number of worms responding and the extent of contractions (*i.e.*, the withdrawal in centimeters), the habituation rate (r) has been computed for populations of worms from the following formula:

$$r = \frac{T}{15}$$

where T is the trial at which 50 per cent of the total withdrawals, measured by the extent of contraction, have occurred. The rate of habituaion is related to the value of r: small values indicate rapid habituation; values larger than one indicate that habituation is not occurring at all.

RESULTS

Rates of habituation and response frequencies of intact worms in tubes

Sudden increases in illumination elicit slower withdrawal responses, with longer latencies, than the other stimuli used. These all elicit the characteristically rapid withdrawal responses, which occur to sudden decreases in illumination in *N. diversicolor* (Evans, 1969). However the manner in which habituation occurs is not always the same: initially shadows, sudden decreases in illumination and mechanical shocks elicit extensive withdrawals, which are almost immediately replaced by incomplete contractions, whereas the initial extensive withdrawals to tactile stimuli and sudden increases in illumination are gradually lost with successive trials.

A worm's response frequency and the rate at which it habituates to a particular stimulus are usually related. Worms which respond infrequently to a stimulus normally habituate rapidly to it and, conversely, reactive worms usually habituate slowly. But this is not always so; *N. pelagica* responds infrequently to anterior tactile stimuli by withdrawing but habituates relatively slowly to them (Table II).

There is some evidence to support the view that the rate of habituation and also the reactivity of the worms can be related to the severity or significance of the stimulus. Habituation is slower and worms respond more frequently to stimuli which are most likely to act as token stimuli warning them of the approach of a predator. For instance paired stimuli are more likely to be related to the approach of a predator, and are therefore more significant, than stimuli presented alone,

TABLE II

Response frequencies and rates of habituation of intact and decerebrate worms in tubes

	Stimulus						
	Light on	Light off	Moving shadow	Mechan. shock	Light off and mech. shock	Ant. tactile	Post. tactile
	Response frequencies						
Intact *N. diversicolor*	19.4	5.6	4.7	5.1	14.8	26.2	29.9
Decerebrate *N. diversicolor*	6.0	3.5	2.3	3.4	9.8	26.3	29.0
Intact *P. dumerilii*	0.7	3.2	4.0	1.5	10.2	23.2	30.0
Decerebrate *P. dumerilii*	2.2	1.8	0.9	1.8	6.2	29.6	30.0
Intact *N. pelagica*	8.4	7.2	7.8	4.9	6.3	2.2	18.7
Decerebrate *N. pelagica*	0.9	0.8	2.9	2.6	2.2	6.9	12.0
	Habituation rates						
Intact *N. diversicolor*	0.83	0.13	0.13	0.33	1.00	0.80	0.93
Decerebrate *N. diversicolor*	0.40	0.40	0.73	0.30	0.87	0.87	0.93
Intact *P. dumerilii*	0.07	0.13	0.33	0.07	0.73	0.87	0.93
Decerebrate *P. dumerilii*	0.20	0.13	0.13	0.13	0.40	0.73	0.80
Intact *N. pelagica*	0.67	0.27	0.40	0.20	0.40	0.60	0.60
Decerebrate *N. pelagica*	0.33	0.07	0.33	0.47	0.60	0.40	0.40

because a moving predator necessarily creates a complex of stimuli whereas most innocuous agents do not. Both *P. dumerilii* and *N. diversicolor* react significantly more frequently ($P < 0.01$) (Table II), habituate more slowly (Table II) and contract more extensively (Table III) to the simultaneous presentation of sudden decreases in illumination paired with mechanical shocks than to either stimulus presented alone, although in *N. pelagica* there are no significant differences in performance. Similarly tactile stimuli are considered to be more urgent than sudden decreases in illumination, shadows and mechanical shocks because they indicate contact with the stimulating agent and, except in the case of anterior tactile stimuli to *N. pelagica,* worms habituate more slowly, are more reactive and contract more extensively to them than to these other stimuli (Tables II and III). The behavior of *N. pelagica* towards an anterior tactile stimulus is exceptional; initially it normally withdraws but thereafter it responds only spo-

TABLE III

Mean contractions (cm) to different stimuli

Stimulus		Changes in illumination			Mechanical shocks	Mech. shocks and light off	Tactile stimuli	
Species		Light on	Light off	Shadows			Anterior	Posterior
		Worms in tubes						
N. diversicolor	intact	1.08	0.38	0.41	0.31	0.79	2.03	3.34
	decerebrate	0.31	0.20	0.13	0.26	0.64	3.46	3.89
P. dumerilii	intact	0.06	0.34	0.48	0.23	1.16	2.00	2.77
	decerebrate	0.01	0.21	0.08	0.19	0.68	3.01	4.19
N. pelagica	intact	0.75	0.41	0.51	0.39	0.51	0.25	1.82
	decerebrate	0.07	0.08	0.23	0.19	0.19	0.46	0.97
		Worms not in tubes						
N. diversicolor	intact	0.01	0.02	0.00	0.35		1.53	4.50
	decerebrate	0.00	0.00	0.05	0.05		1.25	2.78
P. dumerilii	intact	0.00	0.01	0.04	0.54		0.77	4.05
	decerebrate	0.01	0.01	0.03	0.08		0.75	4.02
N. pelagica	intact	0.34	0.00	0.00	0.03		0.10	0.87
	decerebrate	0.05	0.02	0.01	0.07		0.38	0.78

radically in this manner; more frequently it responds by attacking the seeker and grasping it with its jaws. *N. diversicolor* and *P. dumerilii* both react by withdrawing to an anterior tactile stimulus in significantly more trials than *N. pelagica* ($P < 0.001$), and contractions are more extensive (Table III). However, like *N. pelagica*, habituation to this stimulus is slow (Table II) and sometimes, late in the habituation process, individuals of both *N. diversicolor* and *P. dumerilii* have been observed to attack the seeker.

The differences in behavior towards anterior tactile stimuli can probably be related to the feeding habits of the worms. *N. diversicolor* and *P. dumerillii* are both primarily herbivorous (Harley 1956; Herpin 1925) and react to anterior tactile stimuli in the same manner as other stimuli which are likely to be associated with predators, that is, by withdrawing. However *N. pelagica* feeds largely on animal food; bivalves, gastropods, crustaceans, bryozoans, sponges and hydroids have all been identified in its diet (Rauschenplat, 1901; Blegvad, 1914; Harley, 1956). Some of this food is presumably taken alive, so that slow habituation is advantageous to the worm, because it means that there is no rapid loss of sensory information that might indicate the presence of prey and, as withdrawal is incompatible with this type of feeding behavior; it is equally important that the withdrawal response is relatively unreactive.

There is no obvious relationship between the response frequencies and rates of habituation of the withdrawal response and the normal background of stimulation experienced by the worms. For example, the rates of habituation and response frequencies cannot be related to the worms' normal experience of wave action. Species normally exposed to waves might be expected to habituate rapidly to mechanical shocks because this stimulus occurs innocuously with each in-coming and out-going tide but, in fact, *N. diversicolor* and *N. pelagica*, which were collected from exposed shores at Millport, are significantly more reactive ($P < 0.05$) and habituate more slowly to this stimulus than *P. dumerilii*, which was collected sub-littorally, where it is not exposed to wave action. Similarly in intact worms there are no clear differences in the response frequencies ($P > 0.05$) or rates of habituation to sudden decreases in illumination and shadows in *N. diversicolor*, collected from a habitat free from agents, such as seaweed, which are likely to cause innocuous shadows, *P. dumerilii*, from a depth of 20 m where there is only dim illumination, and *N. pelagica*, collected from a rocky shore where there are such dense growths of seaweed that animals living there must be continually bombarded by shadows (Table II).

The only light stimulus to which habituation rates and response frequencies differ substantially is a sudden increase in illumination. *P. dumerilii* hardly responds to this stimulus at all; it responds significantly less frequently ($P < 0.01$) than either *N. diversicolor* or *N. pelagica*, whose reactivity is intermediate between that of the other species and significantly different from both of them ($P < 0.01$) (Table II). There are similar relationships between the rates of habituation (Table II) and mean extent of contractions (Table III) to sudden increases in illumination in these worms.

P. dumerilii is the only sub-littoral nereid of the three species studied, and its behavior towards sudden increases in illumination appears to resemble that of other sub-littoral polychaetes, such as *Branchiomma vesiculosum* (Nicol, 1950), *Hydroides dianthus* (Yerkes, 1906) and *Serpula vermicularis* (Hess, 1914), which have well developed shadow reflexes but do not respond at all to sudden increases in illumination. A sudden increase in light intensity cannot be related to the approach of a predator and is probably only caused innocuously by, for example, water currents and the emergence of the sun. It is not, therefore, likely to be a warning stimulus so that failure to respond to it or very rapid habituation is clearly advantageous.

Although the littoral nereids *N. pelagica* and *N. diversicolor* habituate slowly to sudden increases in illumination, responses to this stimulus have already been shown to differ in several respects from responses to sudden decreases in illumination. They are slower, for instance, and have longer stimulus-response latencies (Evans, 1969). This suggests that the stimulus may not be a warning stimulus at all and the responses not predator-escape responses. Indeed Clark (1960b) has already suggested that the response of *N. pelagica* to sudden increases in illumination may enable it to withdraw from sudden increases in sunlight and to avoid the harmful effects of solar irradiation. However an alternative explanation is necessary for *N. diversicolor* because this worm has often been observed to feed in bright summer sunshine. In fact, Harley (1956) found that it feeds more in light than in darkness, and, as far as can be seen, individuals suffer no ill-effects of solar irradiation. A possible explanation of the behavior to sudden increases in illumination is that the stimulus indicates exposure which would occur if the posterior end of the worm is extended from the burrow or if the substratum is disturbed. Withdrawal is important to the exposed worm, because it is susceptible to attack from a predator but, unless it is accompanied by other stimuli,

TABLE IV

Response frequencies and habituation rates of intact and decerebrate worms not in tubes

	Stimulus					
	Light on	Light off	Moving shadow	Mechan. shock	Ant. tactile	Post. tactile
	Response frequencies					
Intact *N. diversicolor*	0.1	0.1	0	2.6	15.1	29.0
Decerebrate *N. diversicolor*	0	0	0.9	0.6	17.5	25.6
Intact *P. dumerilii*	0	0.1	0.3	3.0	10.0	28.8
Decerebrate *P. dumerilii*	0.1	0.2	0.3	0.9	12.8	29.5
Intact *N. pelagica*	5.3	0.1	0	0.3	1.5	9.0
Decerebrate *N. pelagica*	0.8	0.3	0.2	0.8	5.2	10.2
	Habituation rates					
Intact *N. diversicolor*	0.07	0.13	0	0.40	0.40	0.80
Decerebrate *N. diversicolor*	0	0	0.07	0.13	0.73	0.93
Intact *P. dumerilii*	0	0.07	0.20	0.40	0.87	0.93
Decerebrate *P. dumerilii*	0.07	0.07	0.13	0.13	0.53	0.87
Intact *N. pelagica*	0.93	0.07	0	0.07	1.00	0.87
Decerebrate *N. pelagica*	0.13	0.07	0.07	0.13	0.93	0.60

warning of the approach of a predator, the slow withdrawal response which occurs to sudden increases in illumination in the laboratory would be adequate.

Rates of habituation and response frequencies of intact worms not in tubes

Nereids are often considered to be typically "errant" polychaetes, and although this view is unjustified because most species are burrowing or tubiculous animals, there is no doubt that they are capable of crawling and swimming outside their tubes or burrows. The extent to which nereids leave their tubes or burrows is unknown, but *N. diversicolor* has occasionally been seen crawling across mud, and *N. pelagica* is frequently found beneath stones under which there is no evidence of a tube. Many species leave their tubes in order to reproduce, and these include *N. diversicolor* which copulates on the surface mud (references in Clark, 1961), and *P. dumerilii* which swarms at the surface of the sea (Sorby, 1906); the breeding habits of *N. pelagica* do not appear to have been recorded.

Except in the case of responses of *P. dumerilii* to mechanical shocks, worms outside their tubes respond much less frequently to stimuli than worms in tubes (Table IV; *cf.* Table II). Similarly habituation rates are faster in worms outside their tubes, except to sudden increases in illumination and tactile stimuli for *N. pelagica* and mechanical shocks for *N. diversicolor* and *P. dumerilii*. In general, the differences in performance are relatively large to light stimuli and small to tactile stimuli.

The response frequencies and habituation rates of nereids in these experiments can probably be related to the importance of the withdrawal response to worms outside their tubes or burrows. It can no longer effect escape from predators and in general, therefore, responses to stimuli which might be caused by predators are no longer advantageous, particularly as withdrawal usually inhibits movement. However tactile stimuli may be of significance to worms outside tubes or burrows; withdrawals of the anterior end function as avoiding reactions to solid objects, and posterior end withdrawals to tactile stimuli are usually followed by accelerated movement away from the source of stimulation so that, unlike withdrawals to other stimuli, they may be effective in aiding escape from predators. If this is so it is not surprising that the worms continue to habituate slowly and react frequently to tactile stimuli when they are not confined to tubes.

The effect of decerebration on the rates of habituation and response frequencies

Decerebrate worms react to stimuli in the same manner as intact ones except that irrigating movements are only inhibited while a worm is in the process of contracting; decerebrate worms irrigate incessantly. It is surprising that the worms respond to changes in illumination after removal of the supra-esophageal ganglion because the operation involves removal of the eyes, which are situated immediately dorsal to the ganglion. Light receptors are probably located in the epidermis of the body wall and, in fact, Langdon (1900) identified some spiral organs in the epidermis of *N. virens* as light receptors.

Removal of the supra-esophageal ganglion has apparently little effect on the rates of habituation and response frequencies to different stimuli. Without the ganglion, worms still habituate to repeated stimulations, and similar relationships exist between the rates of habituation and response frequencies to stimuli to those in intact animals (Tables II and IV). For example, both decerebrate and intact worms out of tubes are generally less reactive to anterior and posterior tactile stimuli than worms in tubes.

In most cases decerebrate worms respond less frequently and habituate more rapidly than intact ones, but this can probably be attributed to the effects of the anesthetic and operation, which invariably involves the loss of some blood and coelomic fluid. There are, however, two notable exceptions in which decerebrate worms are either more reactive or habituate more slowly than intact worms. First, decerebrate worms respond more frequently than intact ones to anterior tactile stimuli (Tables II and IV), but, although care was taken not to stimulate the wound, this may be due to increased sensitivity at the anterior end as a result of the operation and, in fact, in most cases these worms habituate more rapidly than intact ones. Secondly, decerebrate *N. diversicolor* in tubes habituate more slowly to shadows and sudden decreases in illumination than intact worms (Table II). However, in this case the decerebrate worms actually responded less frequently than intact worms but habituated in a different manner from them. Decerebrate worms did not contract as extensively to the initial stimulations as intact worms, and therefore the loss of the response was gradual (*i.e.,* slow habituation) whereas intact worms habituated characteristically; there was a sudden loss of the initial extensive contraction (*i.e.*, rapid habituation) (Fig. 1). In fact, both decerebrate and intact worms continued to respond for about the same number of trials to these stimuli.

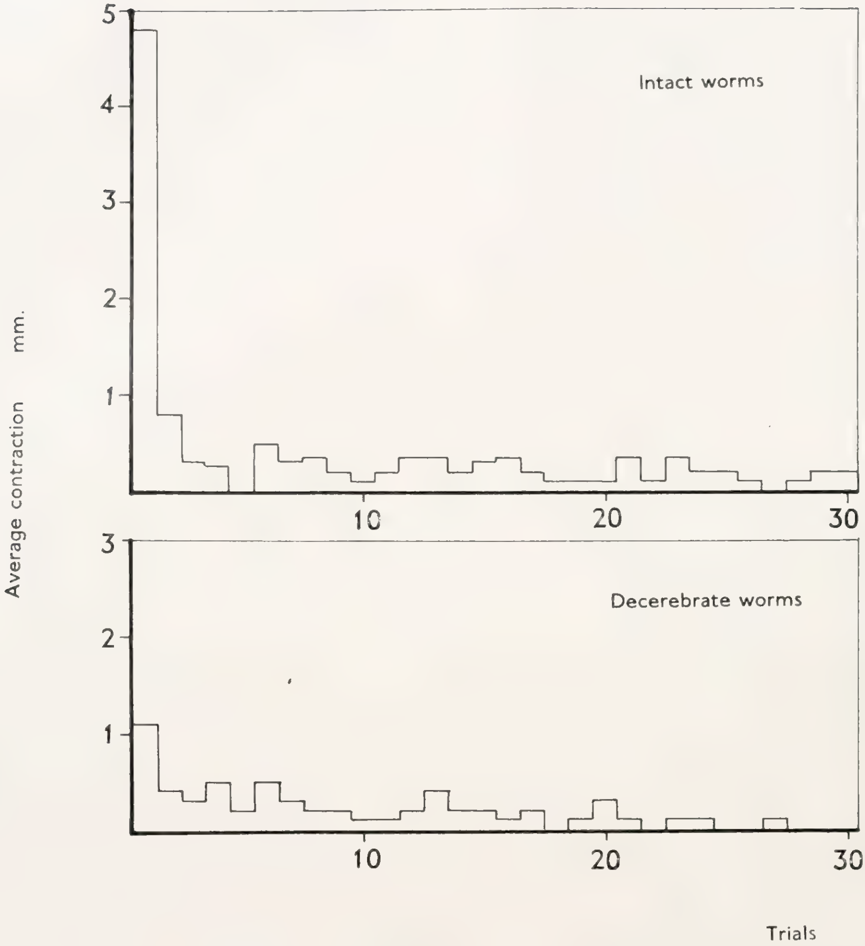

FIGURE 1. Habituation of 12 intact and 10 decerebrate *N. diversicolor* to sudden decreases in illumination.

DISCUSSION

The process of habituation in *Nereis* is not dependent upon the presence of the supra-oesophageal ganglion. Withdrawal is still elicited by stimuli in decerebrate worms, and there are similar relationships between the rates of habituation to different stimuli in intact and decerebrate worms.

Habituation has already been demonstrated in both vertebrates and invertebrates deprived of the higher centers of the central nervous system. For example, spinal rats (Prosser and Hunter, 1936) are capable of habituating. In the octopus the situation is less clear. Boycott (1954) has shown that octopuses lacking all of the higher centers but the optic lobes, which are necessary for sight, will habituate to a moving card in the visual field. Initially the animals withdraw but soon come to ignore the stimulus. Habituation is occurring in either the optic lobes or the lower centers of the nervous system. However, in another situation the higher centers are definitely necessary for habituation. Wells (1961) found that, after a few trials, intact octopuses would learn not to respond to an inedible cylinder by taking it to the mouth, but this type of habituation did not usually occur in animals with inferior lobe lesions.

Young (1961) recognizes two types of habituation, apart from the extinction of conditioned responses. First, there is the habituation of inborn avoidance responses and secondly, the loss of exploratory responses to moving objects. In the two invertebrates so far studied, *Nereis* and *Octopus,* higher centers are not apparently necessary for the habituation of avoidance responses, but, as stated above, they are necessary in *Octopus* for habituation of an exploratory response to an inedible cylinder. However, in the latter case the learning may be more complex than the habituation of an avoidance response, because it involves not only the failure of an exploratory response, but the acquisition of a rejectory response; the cylinder is actively rejected by the octopus. The learning may be more similar to associative learning than habituation, and it is interesting that the inferior lobes, which are necessary for the octopus to learn to reject the cylinder, are also the centers of associative tactile learning.

There is little doubt that the reactivity and habituation rate of the withdrawal response in nereids and other polychaetes is related, in at least some ways, to the significance of the stimulus. For example, sudden increases in illumination, which cannot be related to the approach of a predator, never elicit withdrawal in *Branchiomma vesiculosum* (Nicol, 1950) and, although *P. dumerilii* sometimes responds to this stimulus, it habituates to it extremely rapidly. Stimuli which presumably act as token stimuli warning the animals of the approach of a predator are naturally not all of the same significance. There is a hierarchy of stimuli to which the worms are less and less reactive and to which they habituate more and more rapidly which can probably be associated with the likelihood of these stimuli representing predators under natural conditions. Paired stimuli are presumably more significant than the constituent ones presented alone, because moving predators necessarily cause a simultaneous complex of stimuli, and it is now known that *Mercierella enigmatica* (Rullier, 1948), *N. pelagica* (Clark, 1960b) and in the present work, *N. diversicolor* and *P. dumerilii,* habituate more slowly to paired stimuli than single ones. Similarly tactile stimuli are significant, because they indicate contact with the stimulating agent, and worms, such as *Branchiomma vesiculosum* (Nicol, 1950; Krasne, 1965), *Hydroides dianthus* (Yerkes, 1906) and *N. diversicolor* and *P. dumerilii,* habituate extremely slowly to them. *N. pelagica* is an exception in this respect, because it attacks the seeker by grasping it with its jaws more often than it withdraws when stimulaed by it. However, *N. pelagica* is carnivorous so that it seems likely that this behavior can be related to the worm's feeding habits. *P. dumerilii* and *N. diversicolor* are both herbivorous, and, although they have both been observed to attack the seeker occasionally, they only do so after a long series of trials.

The effect of environmental experience on the rates of habituation and reactivity to different stimuli is obscure. *N. diversicolor, N. pelagica* and *P. dumerilii* are found in entirely different ecological situations, but their rates of habituation and response frequencies to stimuli are remarkably similar. However, unless differences in this kind of comparison are particularly clear their significance can only be assessed with suspicion. In order to assess the significance of a particular stimulus in different habitats the important consideration is the relative preponderance of that stimulus caused by innocuous and harmful agents in each habitat, and there are probably insuperable difficulties in obtaining such information.

It is not clear from the data whether the rate of habituation to a particular stimulus is innately determined in nereids, and modified only as a result of recent experience, or whether it is adapted to the background of stimulation experienced over a longer period of time. The latter situation is advantageous if individuals of a species are likely to occur in different habitats in which selective pressures vary to such an extent that stimuli do not have the same significance in them. However, nereid species are usually found in well defined habitats, and it may be that habituation rates are innately determined in this animal.

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SUMMARY

1. Rates of habituation and response frequencies have been compared in *N. diversicolor, P. dumerilii* and *N. pelagica* to changes in illumination, tactile and mechanical stimuli. In general habituation is rapid to light stimuli and mechanical shocks and slow to tactile stimuli.

2. Worms in tubes are generally more reactive than worms not in tubes.

3. Decerebrate worms respond to the same stimuli as intact ones. They also habituate to them, and similar relationships exist between the response frequencies and rates of habituation to different stimuli in intact and decerebrate worms.

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INTESTINAL ABSORPTION AND TRANSPORT IN *THYONE*.[1]
I. BIOLOGICAL ASPECTS

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During the first three decades of this century several investigators reported controversial observations about the digestion of food and the intestinal transport of nutrients in holothurians. These reports have been reviewed by Anderson (1966). Briefly, Enriques (1902), Oomen (1926), and Schreiber (1931) reported that the intestine of *Holothuria* is impermeable to a variety of sugars, salts, and dyes. These authors proposed that the distribution of nutrients from the intestinal lumen to the various tissues of the body is mediated by wandering coelomocytes which shuttle between the intestinal lumen and the different tissues and body fluids of holothurians. This view was based only upon the observation that coelomocytes are found in histological preparations of all holothurian tissues. While amebocytic transport of this type probably plays a substantial role in nutrient distribution among animals which do not possess well differentiated digestive tracts, it is difficult to accept the contention that such a mode of nutrient transport is effectively operative in echinoderms with well developed digestive systems. Some support for the coelomocyte theory might be adduced from the observation of direct uptake of dissolved sugars and amino acids from surrounding sea water. This has been demonstrated for various echinoderms by Koller (1930), Stephens and Schinske (1961), and Ferguson (1967). However, this mode of nutrition does not appear to supply these animals with a significant number of calories. Echinoderms are known to feed on a variety of materials in nature; their digestive tracts are usually found to be packed with specific materials. When deprived of solid or particulate food, they starve; there is a rapid drop in metabolic rate and body reserves. For the pertinent data see Farmanfarmaian (1966), Giese (1966a), Holland, Giese and Phillips (1967) and Lawrence, Lawrence and Holland (1965).

In studies on the transport of nutrients in echinoids, Farmanfarmaian and Phillips (1962) demonstrated that galactose is directly transported across the intestinal wall without appreciable involvement of coelomocytes. Ferguson (1964) used C^{14} labeled glucose and amino acids and reached the same conclusion for asteroids. The holothurian digestive tract is well developed and usually longer than those of echinoids and asteroids (Choe, 1962). It seemed unlikely that this extensive intestine should be impermeable to digested nutrients as was claimed by the early authors. These considerations led to preliminary investigations of the transport of glucose across the intestine of *Leptosynapta* by D'Agostino and

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Farmanfarmaian (1960); of *Holothuria* by Farmanfarmaian (1963); and of *Thyone briareus* by Rundles and Farmanfarmaian (1964). The intestinal wall of all three species were shown to be permeable to glucose but these exploratory studies did not reveal any details of the mechanisms of nutrient absorption and transport in holothurians. Furthermore, the cellular mechanisms involved in the intestinal transport of most invertebrates have not been adequately investigated. By contrast, the mammals have been extensively studied in this respect. For recent reviews of the mammalian literature see Wilson (1962), Wiseman (1964), and Crane (1968).

The above considerations prompted a detailed *in vivo* and *in vitro* investigation of the intestinal absorption and transport of nutrients in *Thyone briareus*. Since this organism is not a standard laboratory animal, much of the biological information relevant to the mode of nutrition and the mechanisms of absorption and transport had to be determined as part of the present project. This paper is devoted to the presentation of this biological background.

MATERIALS AND METHODS

Animals

Specimens of *Thyone briareus*, weighing 25–50 g, were obtained from the Supply Department of the Marine Biological Laboratory on the day of collection. All the animals used in these investigations were collected between June 10 and September 10. The animals in each collection were kept at about 20° C in separate shallow tanks of running sea water without special feeding. To circumvent the effects of the duration of captivity on various experiments, only animals from the same collection, and with similar background, were used for comparable experiments.

The dissection procedure was as follows: The animal was lightly anesthetized in 7% $MgCl_2$ tap water solution for 40 minutes and thoroughly rinsed in sea water. The relaxed animal was placed on a shallow dissecting pan and a postero-anterior incision made along the convex curvature of the body wall. The incision was extended laterally near the anterior and posterior ends and the body wall was pinned out under 1.5 cm of sea water. Under these conditions the animal was completely immobilized although the effect of anesthesia had worn off. In these preparations vital functions such as the movements of the gut and the branches of the water lungs could be observed for several hours at 20° C.

Chemical analyses

Glucose was measured colorimetrically by the glucose oxidase method (Glucostat and Glucostat Special from the Worthington Biochemical Corporation, Freehold, New Jersey). A Bausch and Lomb Spectronic 20 was used for this purpose. The precision of the method was ±2% in the range of 5–30 μg per sample. Total carbohydrates were measured according to the anthrone method of Koehler (1952) and the values are expressed in terms of glucose equivalents. Glycogen was purified according to Be Miller (1965) and analyzed by the anthrone method.

For thin layer chromatography (TLC) of sugars, Eastman cellulose sheet number 6064 and developing apparatus number 6071 were used. Solutions containing 1–2 μg of sugar were spotted on the sheet about 3 cm apart and the chromatogram was developed in ethyl acetate:pyridine:water (120:50:40 vol.) at 23° C. The TLC sheets were visualized by the $AgNO_3$ and NaOH dip method of Smith (1960). For desalting prior to chromatography, 1–2 volumes of wet ion exchange resin (Rexyn 300-H-OH Research Grade, Fisher Scientific Company) was mixed with the sample in a centrifuge tube for 2–3 minutes. The fluid was aspirated and the resin washed twice with distilled water and the washes added to the aspirated fluid. This procedure gave 70–75% recovery of sugars without detectable salts. Further washes increased the recovery to better than 90%. When desired the desalted fluid was concentrated under vacuum at 40° C.

Total lipid was determined by a modification of the method of Freeman, Lindgren, Ng and Nichola (1967). Tissue samples of 4–5 g fresh weight were extracted in 50 ml methanol at 65° C for 10 minutes. At room temperature, 100 ml of chloroform was added to the mixture and the extraction was continued under constant stirring for 4 hours. The extract was filtered and passed through a 10 cm column of Unisil (silicic acid). This was followed by elution with 100 ml of 95% methanol and 5% water to obtain all the phospholipids. The eluates were combined and concentrated. Aliquot portions were dried in tared glass tubes under nitrogen and weighed.

Water content of intestinal tissues were determined as the difference between the wet weight and the constant dry weight measured by a Mettler analytical balance. The wet weight was measured after the tissue was allowed to drain on a piece of Whatman No. 1 filter paper for 30 seconds.

For pH measurements a Beckman Zeromatic pH meter was employed.

Histological preparations

Tissues were fixed in Bouin sea water fixative. Sections of 10 μ thickness were stained with iron hematoxylin or Mallory's triple stain. For electron microscopy tissues were fixed in 6% glutaraldehyde, postfixed in osmium tetroxide, and embedded in maraglass or Epon 812. These sections were cut at 0.5 μ and a few were stained with methylene blue Azure B for light microscopy.

RESULTS

Ecological observations

In the Woods Hole region *Thyone briareus* is found partially buried among the eel grass in sallow bays. In the contracted state the size of the mature specimens may vary from 4 to 5 cm in length. The ambient temperature of its environment, as reported by Mr. John Valois, Chief Collector of the Supply Department, varies from 1° C in winter to 22° C or more in the summer. The sexes are separate and the gametes are usually spawned out by the end of June. These animals are particle feeders, using their tentacles as a filtering mechanism. Feeding activity has not been observed during winter when *Thyone* appears to be in a state of hibernation.

In the laboratory the animals survive well for more than three months at about 20° C in shallow tanks of running sea water without special feeding. Attempts at controlled feeding with sea water species of *Chlorella* have failed. *Thyone* can tolerate salinities as low as 50% sea water and as high as 120% sea water for 24 hours or more.

During the summers, when the investigations to be reported here were carried out, the contents of the digestive tract largely consisted of particles of the eel grass *Zostera*. When animals are brought into the laboratory, the gut is emptied within a week and the feces consist of greyish masses of unrecognizable amorphous materials which disintegrate at the bottom of the tank.

The carbohydrate composition of various species of *Zostera* has been determined by Dudkin, Shkantova, Skornyakova and Lemle (1964) and Williams and Jones (1964). This sea grass is known to contain glucose, fructose, galactose, xylose, arabinose, rhamnose, and apiose. The natural gut fluid of *Thyone* was analyzed for glucose in the following manner. The intestinal contents taken from 15 animals (freshly collected during July) were pooled and centrifuged at 5° C. The iced supernatant was deproteinized with $Ba(OH)_2$ and $ZnSO_4$, decolorized with norite, and the pH adjusted to 7.0. Samples were analyzed by the highly purified glucose oxidase (Glucostat Special). The glucose content was 33.2 μg/ml of the original intestinal fluid. Samples analyzed for total carbohydrates by the anthrone method gave a glucose equivalent value of 66.7 μg/ml for freshly collected animals and 31.2 μg/ml for animals starved for 24 hours in the laboratory. These results serve to indicate the general level of dissolved sugars in the natural intestinal fluid of *Thyone*.

Anatomical and histological observations

Figure 1 illustrates a freshly dissected digestive tract laid out in an approximately natural disposition. Various nomenclatures have been applied to the different regions of the digestive tract of holothurians (Fish 1967a). In the case of *Thyone*, the digestive tract consists of an oropharynx, a short esophagus which goes through the calcareous aquapharyngeal bulb, a yellowish bulbous stomach, and a long brownish red intestine which terminates in the cloaca. The intestine constitutes 3–4% of the animal fresh weight. For the purpose of the physiological studies, the intestine has been divided into zones which are anatomically recognizable (Fig. 1). These zones are:

First loop—from the posterior end of the stomach to the last posterior cross connecting hemal sinus of the hemal network.

Clear zone—the segment between the first and second loop which has no interconnecting hemal sinuses to the hemal network.

Second loop—the segment between the first (anterior) and the last (posterior) cross connecting hemal sinuses of the network on the ascending part of the intestine.

Cloacal segment—the remaining part of the intestine which terminates in the cloaca and has been referred to as the large intestine in the literature. The intestine of animals which have eviscerated and are in the process of regenerating the intestine does not precisely conform to the above anatomical description.

Histological sections of the intestine were examined by light microscopy and low magnification electron microscopy.

From the lumen outward, the intestinal wall typically consists of tall (100–180 μ long) mucosal epithelial cells with a brush border (Figs. 2 and 3). These cells

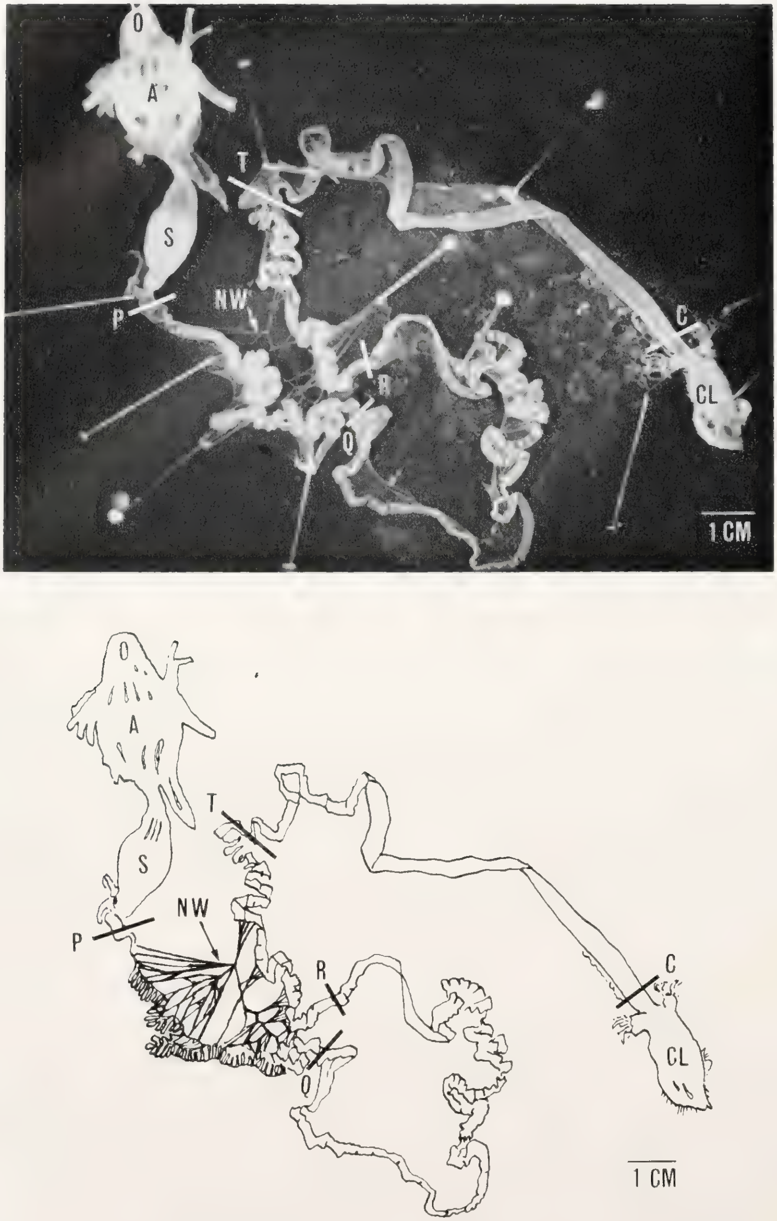

FIGURE 1. Freshly dissected digestive tract of *Thyone* laid out in approximately natural size and disposition. O-oropharynx; A-aquapharyngeal bulb containing the esophagus; S-stomach; P to Q-first loop; Q to R-clear zone; R to T-second loop; T to C-cloacal segment; CL-cloaca; NW-net work of hemal sinuses interconnecting the first and the second loop.

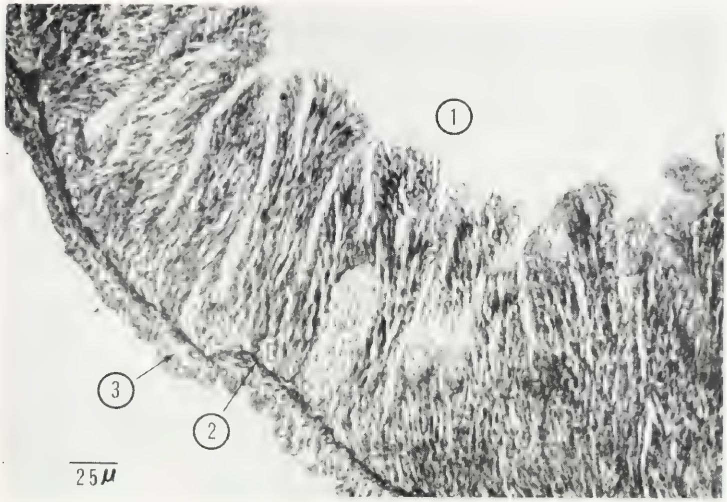

FIGURE 2. Cross section near the anterior end of the first loop showing the brush border of the tall mucosal epithelium extended into the lumen (1); circular muscle bands (2); and the flat serosal epithelial cells (3). Fixed in Bouin and stained with iron hematoxylin.

are loosely organized into villus like structures which make up the bulk of the intestinal tissue (Fig. 2). At the base of the epithelial cells is the connective tissue-lacunar area (10–30 µ wide) with mixed connective tissue fibers and a fluid which has the same staining properties as the clear material in the mesenterial sinus (Fig. 4). This fluid is in communication with the hemal sinuses (Figs. 4 and 5). The connective tissue-lacunar areas invade the base of the "villi" and break up into fine channels (Fig. 4), somewhat akin to the lymphatic channels of the vertebrate villi (Bell and Farmanfarmaian, 1967). Toward the outside there is a longitudinal muscle layer followed by a circular muscle layer (Fig. 5). These muscles are particularly prominent in the cloacal segment of the intestine (10–15 µ). Peripheral to the muscles is a thin connective tissue layer and finally the flat serosal epithelium (10–30 µ) bearing a few observable cilia (Fig. 5).

The intestine is attended by a mesenterial and an antimesenterial hemal sinus along its length (Figs. 4 and 5). The side channels of these sinuses invade the serosal epithelium and open into the connective tissue-lacunar area below the mucosal epithelium. There are numerous interconnecting hemal sinuses forming a network between the antimesenterial sinus of the first loop and the second loop (Fig. 1). The anatomical relation of the hemal sinuses to the intestine would seem to indicate that the hemal sinuses provide for a functional circulation of the absorbed nutrients. In surviving preparations, which were dissected as described in a previous section, there was no observable unidirectional Harveyan flow in these sinuses. Weak local contractions provided for gentle ebb and flow movements of the viscous contents of the channels. By contrast, vital functions such as the movement of sea water in and out of the branches of the water lungs, the circulation of the hemoglobin-containing hemocytes in the ampullae of the podia, and the peristalsis of the gut were all clearly observable for several hours in such a

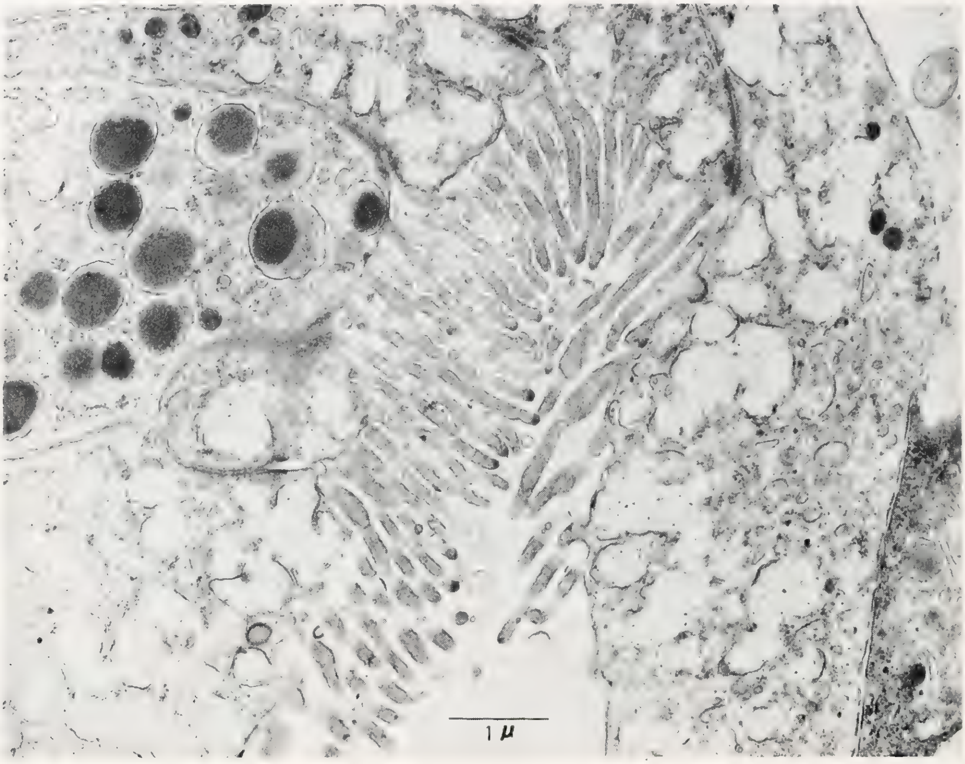

FIGURE 3. Electron micrograph showing the brush border "microvilli" of the mucosal epithelial cells. Photograph kindly provided by Dr. Allen Bell. See text for methods of preparation.

preparation. There are no functional hearts of any nature in holothurians. In some species, however, more prominent local contractions can be observed in the mesenterial sinuses. For example, in preparations of *Leptosynapta* a vigorous churning motion was observed in the mesenterial sinus which is tightly applied to the wall of the gut. This motion, however, was caused by the peristaltic waves of the intestine and had the same rhythmicity. The viscous yellowish fluid in the mesenterial sinus moved back and forth and there was no evidence for its circulation. When the hemal sinuses of *Thyone* and *Leptosynapta* were cut, the hemal fluid did not spurt. Either there was no flow from the cut end or only a small amount of the viscous yellowish fluid slowly leaked out. The author has also examined many other American and European echinoderms in this connection. In no case has he observed a unidirectional Harveyan circulation in the hemal system of echinoderms.

The total weight of the full (undrained) network sinuses in a 50 g *Thyone* is about 2 mg. This represents roughly 25% of the entire hemal system of the animal and indicates the low capacity of this system, 0.02% of the body weight.

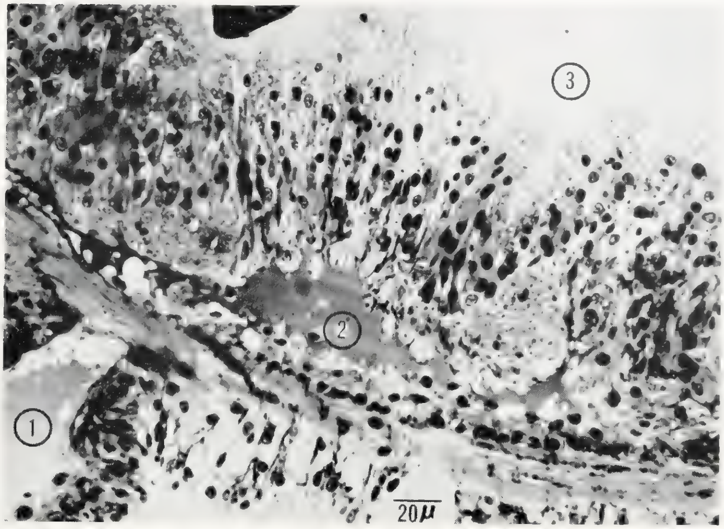

FIGURE 4. Cross section of the first loop cut at 0.5 μ. The side channel of the hemal sinus (1) is shown entering the serosal epithelium. Small channels of the connective tissue-lacunar area (2) penetrate between the bases of the mucosal epithelial cells. Lumen (3). Photograph kindly provided by Dr. Allen Bell. See text for methods of preparation.

Hemal and perivisceral fluids

It has not been possible to obtain samples of the content of hemal sinuses without contamination and in adequate amounts for analysis. When a portion of fresh hemal sinus is smeared on a microscope slide, examination shows that it contains yellow, brown, and black refractile spherules. These bodies show autofluorescence under ultraviolet light and in this respect are similar to the lipofuscin granules described by Goldfischer, Villaverde and Forschirm (1966). Histochemical studies of the hemal sinuses of *Thyone* (Hugh Y. Elder, personal communication) showed that the clear fluid is PAS positive but Alcian blue and hemalum negative. These reactions indicate a large amount of dissolved neutral polysaccharides or possibly non-sulphated mucopolysaccharides or glycoproteins. The lining of the sinuses, however, contains acid mucopolysaccharides. In addition to the clear fluid and lipofuscin spherules, coelomocytes are always observed in various parts of the hemal sinuses.

The perivisceral fluid occupies the large body cavity of holothurians and bathes all the internal organs. A large specimen of *Thyone* (40–50 g fresh) contains about 10 ml of this fluid. The perivisceral fluid contains a variety of coelomocytes (Hetzel, 1965; Endean, 1966). The ionic composition and the osmotic pressure of this fluid are essentially the same as those of the surrounding sea water (Binyon, 1966).

For chemical analysis, perivisceral fluid was obtained by a direct incision of the body wall of *Thyone*. The fluid was pooled from 5 or more freshly collected animals in July. The pooled sample was deproteinized by 10% TCA, its pH adjusted to 7.0 and centrifuged at 5° C. The supernatant was concentrated in a

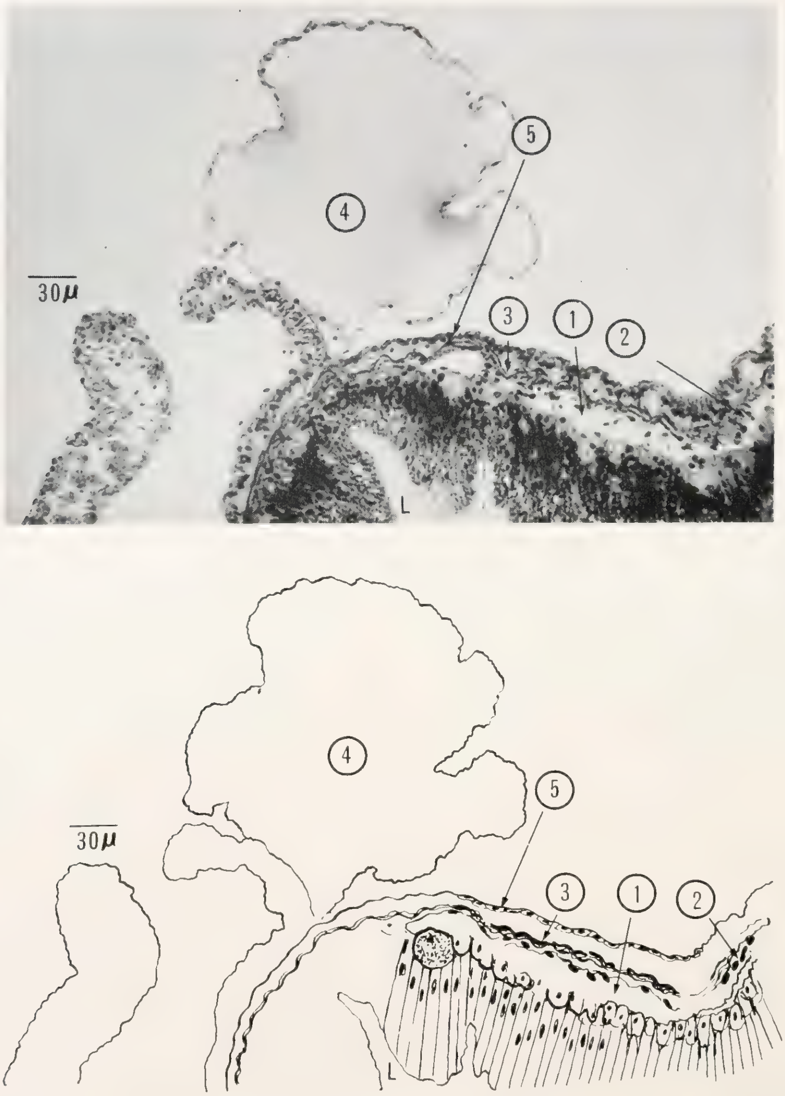

FIGURE 5. Cross section of the clear zone showing: connective tissue-lacunar area (1), longitudinal muscle bands (2), circular muscle bands (3), clear viscous material in the mesenterial sinuses (4), serosal epithelial cells (5), and lumen (L). Fixed in Bouin and stained with Mallory's triple stain.

Buchler evaporator at 40° C. Samples of the concentrates were analyzed by the anthrone method for total carbohydrates and by Glucostat Special for glucose. The values obtained for total carbohydrates were 6.3 and 7.4 μg/ml and those for glucose were 1.2 and 1.5 μg/ml of original fluid. Several sugars were identified by thin layer chromatography of the concentrates. These were glucose, galactose, xylose, and trehalose. Thus, the perivisceral fluid of *Thyone* contains an appreciable amount of dissolved carbohydrates in nature and these include several of the sugars found in the natural diet of the animal.

The pH of the perivisceral fluid measured outside of the body of a number of freshly collected animals varied between 7.3–7.8.

Chemical composition of the intestinal tissue

Intestinal segments from the first loop and the clear zone were removed from freshly collected *Thyone* and both the mucosal and serosal surfaces were washed with cold filtered sea water. These tissues were homogenized in iced 10% TCA. Aliquot portions of the whole homogenate were used to determine total carbohydrate. Other aliquots were neutralized, decolorized by norite, and centrifuged. The supernatant was analyzed for glucose by Glucostat Special. For animals collected in July mean value of total carbohydrate for the first loop was 10.3 mg/g wet (SEM ± 0.5; N = 9) and for clear zone was 13.9 mg/g wet (SEM ± 0.6; N = 9). In the August collection corresponding values were 12.0 and 16.6 mg/g wet. In all of these segments, the free glucose content was below the sensitivity (20 μg/g wet) of the method used. However, by thin layer chromatography it was possible to identify glucose, galactose, and trehalose qualitatively. It appears, therefore, that free sugars occur in small amounts in the intestinal tissue of animals a few hours after collection.

The glycogen content of the first loop and clear zone segments (pooled from 4 animals freshly collected in August) was determined by the anthrone method after two purifications according to Be Miller (1965). The values obtained were 2.7 and 6.2 mg/g wet (or 12.3 and 28.2 mg/g dry) for the first loop and the clear zone, respectively. Glycogen constitutes 22.8% and 37.2% of the total carbohydrate of the respective intestinal segment. The occurence of glycogen, acid mucopolysaccharide, neutral mucoproteins and glycoproteins, and possibly glycolipids has been confirmed histochemically (Hugh Y. Elder, personal communication).

For total lipid determination segments of the first loop and clear zone were separately pooled from 10 animals freshly collected in July. The values obtained were 45.4 and 48.8 mg/g wet (or 207 and 222 mg/g dry) for the first loop and clear zone, respectively.

The mean tissue water for the combined first loop and clear zone segments was 78% (SEM ± 0.48; N = 34) of the wet weight.

DISCUSSION

The digestive tract of *Thyone* is anatomically and histologically well adapted to the digestion and absorption of carbohydrates found in the particles of the eel grass *Zostera* which constitute a large portion of the normal diet of the animal during the summer. The length of the gut (about 50 cm in a 15 cm animal) and the peristaltic and tonic movements of the intestine are appropriately suited to continuous filter feeding. Such an arrangement provides for a large reserve of absorptive surface so that absorption can continue as the intestinal contents are moved distally. The relative weight of the alimentary tract (3–4% of body weight) is appreciably higher than mammals, about 2.3% (Long, 1961). This is probably due to the absence of liver, hepatopancreas, or hepatic caeca in *Thyone*. Most of the hepatic storage functions appear to be retained in the intestinal wall. The tall mucosal epithelial cells constitute the bulk of the intestinal tissue. These cells are 5–10 times taller than the columnar epithelial cells of the mammalian intestine (Bloom and Fawcet, 1962). The luminal borders of the mucosal epithelial cells are invested with an extensive brush border whose "microvilli" appear similar to those of the mammals both in structure and size; approximately 1 μ in length and 0.1 μ in width (Trier, 1968). By analogy to the mammals, the structural evidence indicates that the "microvilli" constitute the normal site for the absorption of nutrients. There is no basis for rejecting this extensive brush border as the absorptive site in favor of a relatively few coelomocytes which are observed in the intestinal tissue. In three different species of holothurians it was experimentally demonstrated that the transfer of glucose from the mucosal to the serosal side of *in vitro* intestinal segments proceeds in the absence of coelomocytes (D'Agostino and Farmanfarmaian, 1960; Farmanfarmaian, 1963; Rundles and Farmanfarmaian, 1964). Comparable data were provided for the intestinal transfer of nutrients in echinoids and asteroids (Farmanfarmaian and Phillips, 1962; Ferguson, 1964). Thus, the coelomocyte theory proposed by the early investigators (Enriques, 1902; Oomen, 1926; Schreiber, 1931) is rejected.

In the mammal, absorbed nutrients are rapidly transferred from the intestine to the lymph and the blood, and from there to the liver and other organs. In *Thyone*, the first site of storage and chemical conversion is the mucosal epithelium. This view is supported by the following lines of evidence: (a) There is no liver-like organ in this animal. (b) The carbohydrate and lipid content of the intestinal tissue indicates that this organ is an important site of reserve material. The total carbohydrate of the intestinal tissue of *Thyone* is about 1/3 and the glycogen about 1/10 of the glycogen values reported by Stimpson (1965) for the liver of rat and goldfish. Nevertheless, this is an appreciable amount of carbohydrate reserve for *Thyone* which has a continuously dependable external source of carbohydrates during the feeding season. The lipid content of the intestine is about 22% of the dry weight while the lipid content of the whole animal is only 1% of the total dry weight (Giese, 1966b). In another holothurian, *Parastichopus*, the lipid content of the intestine was appreciably higher than all other organs (Giese, 1966a). Therefore, the intestinal tissue appears to be the most important site of lipid storage and possibly lipid synthesis. (c) The sea urchin, *Strongylocentrotus purpuratus* is similar to *Thyone* in that it also lacks a liver-like organ and the mucosal epithelial cells constitute the main part of the intestine. When C^{14}-labeled discs of the alga, *Iridaea* (which is equally labeled in its reserve galactose and glycerol) were fed to this sea urchin, the specific activity of the proximal intestine was consistently higher than all other tissues for 7 days after feeding (Farmanfarmaian and Phillips, 1962). The level of activity gradually reduced as the animal was starved and the stored material was mobilized from the intestine for utilization by the other tissues. Lawrence, Lawrence, and Giese (1966) confirmed the above observations in the same sea urchin by reporting that the relative gut size decreases with starvation, primarily as a result of reduction in lipid content. The carbohydrates also declined. Realimentation arrested the decrease and replenished these reserves in the intestine. (d) Finally, *in vivo* experiments on the intestinal absorption and transport of glucose presented in the following paper further support the view that the intestinal tissue is the first site for the storage of absorbed sugar.

The transport of nutrients from the alimentary canal to the other tissues of echinoderms has been a subject of much controversy. Most members of this phylum have three body fluid systems, namely, the water vascular system, the hemal system, and the perivisceral fluid. The last two have been implicated in the transport of nutrients. The evidence favoring the hemal system is based upon anatomy and the observation of local contractions of the sinuses of this system. By contrast nearly all the experimental evidence from studies of respiratory gas transport and nutrient transport indicates that the perivisceral fluid is the functionally effective circulatory fluid of adult echinoderms (Farmanfarmaian, 1968). Among the members of this phylum, the holothurians have the most extensive and the largest hemal systems. Nevertheless, the capacity of the hemal system is negligible when it is compared to the perivisceral fluid or the circulatory fluids of comparable animals. In *Thyone*, the weight of the system with its fluid contents is only 0.02% of the total body weight. By contrast, the perivisceral fluid is approximately 20% of the body weight. The blood volume of poikilotherm vertebrates and invertebrates, whose metabolism is of the same order of magnitude as *Thyone*, is 2-80% of body weight (Prosser and Brown, 1961; Farmanfarmaian, 1966). Therefore, there is good agreement between the capacity of the perivisceral fluid of *Thyone* and the well known circulatory fluids of other metabolically comparable animals. If the circulation in the hemal system were efficient, it might still be assumed that the rapid turn over of a few milligrams of hemal fluid may effectively contribute to the distribution of nutrients since the hemal system is so intimately related to the intestine. In the absence of any kind of a heart or unidirectional valves, there is no Harveyan circulation in this system. Only weak local pulsations of 4–5 beats per minute have been observed in these sinuses (Kawamoto, 1927; Wyman and Lutz, 1930; Prosser and Judson, 1952). On the basis of the available evidence, it is not possible to assign a definite function to the hemal system; it may be vestigial, have an embryonic function, or be involved in the elaboration and slow distribution of some special compounds. The *in vivo* experiments reported in the following paper provide direct evidence for consideration of the perivisceral fluid as the functional circulatory fluid of *Thyone*.

The digestive enzymes of *Thyone* have not been investigated. The simultaneous presence of several sugars including glucose and galactose in the diet, intestinal fluid, intestinal tissue, and the perivisceral fluid indicates that the digestive tract contains the necessary carbohydrases for the digestion of plant particles in the diet. Related species have been investigated in this respect and contain various carbohydrases, proteases and lipases (Fish, 1967b; Choe, 1962). Glucose appears to be the most important sugar in the economy of *Thyone* since it occurs in appreciable quantities in the diet and constitutes half of the total dissolved carbohydrate in the natural intestinal fluid. The concentration of glucose in the intestinal fluid is 20 times higher than the perivisceral fluid. This implies that in nature the transport of glucose across the intestinal wall does not proceed against a chemical gradient. Further studies on the absorption and transport of glucose by the intestine of *Thyone* are reported in the following paper.

I am indebted to Drs. J. M. Anderson and N. Holland for reading and commenting upon this paper.

Summary

Several investigators have proposed that the transport of nutrients from the intestine to the tissues of holothurians is mediated by wandering coelomocytes. These and other authors have also implied that hemal system is a functional circulatory system in adult holothurians. On the basis of the present studies, these views are rejected. Evidence for the direct absorption of glucose by the intestinal epithelium and its transmural transport into the perivisceral fluid is presented. The perivisceral fluid is the main circulatory medium for gaseous exchange and nutrient transport to the internal tissues. Ecological, anatomical, and histological information pertinent to the study of intestinal transport mechanisms is presented. In addition to digestive and absorptive functions, the intestinal tissue serves as the storage site for relatively large quantities of carbohydrate and lipid reserves.

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INTESTINAL ABSORPTION AND TRANSPORT IN *THYONE*.[1]
II. OBSERVATIONS ON SUGAR TRANSPORT

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The biological background for the study of intestinal absorption and transport in *Thyone* was presented in the previous paper (Farmanfarmaian, 1969). This animal has a well developed intestine with tall mucosal epithelial cells. The luminal border of these cells is invested with numerous "microvilli" which are similar to the mammalian intestinal microvilli in size and structure and present an extensive surface for the absorption of nutrients.

A large part of the natural diet of the animal consists of plant particles which contain a variety of sugars. Glucose appears to be the most important sugar in the economy of *Thyone* since it constitutes half of the total dissolved carbohydrates of the natural gut fluid. Free glucose is also found in the intestinal tissue and the perivisceral fluid, the circulatory fluid of this animal. It is therefore the first sugar of choice for studying the intestinal transport of sugars in *Thyone*. The results of investigations of some of the physiological conditions which affect the *in vitro* and *in vivo* absorption, transmural transfer, and distribution of glucose are presented in this paper.

MATERIALS AND METHODS

Animals and tissues

Procedures for the collection, maintenance and dissection of *Thyone* were given in the previous paper (Farmanfarmaian, 1969). For *in vitro* experiments, either the test tube method of Crane and Wilson (1958) or the everted sac method of Wilson and Wiseman (1954) were employed. These methods were slightly modified for the fragile intestine of *Thyone*. For the test tube method, tubes were made from 10 ml pipets with a funneled opening. In these experiments air was bubbled through a fine polyethylene tubing and the gut was suspended from a glass cannula attached to the arm of a micromanipulator. Everted sacs were incubated in Warburg flasks or in open beakers on a gyrotory shaker bath. All intestinal preparations were checked for leaks before and after each experiment and the leaky ones were discarded. In a few experiments leaks were detected by adding enough phenol red to the solution within the sac to make it just visibly colored. Leakage from such sacs into the incubation medium is conveniently observed. When samples of solutions containing phenol red were analyzed, adequate blanks or quench controls were used. In most experiments, however, each ligated sac was placed upon a piece of dry parafilm and its ends alternately lifted to create positive pressure within the sac. Under these conditions, the jet of colorless fluid issuing from leaky preparations in easily detected and the preparation discarded. Unless otherwise indicated, all the experiments were conducted at 20 ± 0.5° C in an air atmosphere and all the solutions were made in isosmotic filtered sea water (FSW) at pH 7.9 ± 0.1. Specific conditions of each experiment are presented with the results.

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Chemical analyses

Procedures for glucose analysis and thin layer chromatography (TLC) of sugars were described in the previous paper. The purity of glucose was checked by TLC.

The amaranth dye dilution method was used to measure changes in the water content of sacs. For this purpose 0.5 mM solutions of amaranth FSW were employed. Samples of 100 μl were obtained at the beginning and at the end of an experiment. After adding 3 ml FSW to these samples the O.D.$_{520}$ was read against a 3.1 FSW blank in the Bausch and Lomb Spectronic 20 colorimeter.

All analyses were in two or more replicates.

Radiotracer procedures

The Packard Liquid Scintillation Spectrometer Model 3375 and radiochromatogram scanner Model 7201 were used for measurements of radioactivity. The procedures employed in the use of these instruments were generally in accordance with those described by Wang and Willis (1965) and the operation manuals provided with these instruments. Radioactive materials were purchased from New England Nuclear Corporation of Boston and the purity of these compounds were checked by TLC visually and by radiochromatographic scanning. Volumes of radioactive samples counted were 10–100 μl as desired. Two or more replicate samples were counted and appropriate blanks, quench controls and standards were included. To each scintillation vial 15 ml of Bray's scintillation fluid was added one hour before the counting. The composition of Bray's fluid was as follows: Napthalene 60 g, Methanol 100 ml, Ethylene Glycol 20 ml, "PPO" 4 g, "POPOP" 200 mg, and enough P-Dioxane to make one liter of fluid. Scintillation chemicals were purchased from the Packard Instrument Company, Downers Grove, Illinois.

Treatment of data

Computations related to the radiotracer method were in accordance with Wang and Willis (1965) or the operation manual of the instruments. Statistical treatment was generally in conformity with those described by Fisher (1958).

Validation of the radiotracer method

In transport studies the radionuclide incorporated into a compound under investigation may appear in other compounds due to exchange or metabolic conversions. Accordingly, quantitative changes in radioactivity may not reflect a proportional change in the quantity of the compound under study. It is therefore necessary to validiate the radiotracer method for each compound and experimental

TABLE I

*Validation of the radiotracer method**

Incubation medium C^{14}-glucose concentration mM	Series A		Series B	
	Glucose oxidase method	Radiotracer method	Glucose oxidase method	Radiotracer method
	$\frac{\mu\text{g absorbed}}{\mu\text{g in initial solution}}$	$\frac{\text{CPM absorbed}}{\text{CPM in initial solution}}$	$\frac{\mu\text{g absorbed}}{\mu\text{g in initial solution}}$	$\frac{\text{CPM absorbed}}{\text{CPM in initial solution}}$
2	$\frac{3.5}{17.5} = 0.20$	$\frac{5975}{31384} = 0.19$	$\frac{7.2}{16.7} = 0.43$	$\frac{12905}{29485} = 0.44$
4	$\frac{1.1}{14.8} = 0.07$	$\frac{1113}{12476} = 0.08$	$\frac{3}{13} = 0.23$	$\frac{2687}{11962} = 0.22$
6	$\frac{1.0}{21.0} = 0.05$	$\frac{786}{13398} = 0.06$	$\frac{3.7}{20.5} = 0.18$	$\frac{1910}{13028} = 0.15$

* Conditions of experiments: Series A—everted sac, proximal clear zone 0.0457 g wet. Series B—everted sac, distal clear zone 0.1564 g wet. Each sac was first incubated in the 2 mM solution, then washed in FSW and reincubated in 4 and then in 6 mM solutions in Warburg flasks. Incubation medium, 1.5 ml of various glucose solutions FSW; serosal medium 0.3 ml FSW; incubation time in each solution 30 minutes. Sample size 50 μl at 2 mM and 20 μl at 4 and 6 mM.

procedure used. This may be done by radiochromatography or by chemical quantitative methods. The latter method was used under actual experimental conditions to validate the radiotracer procedure adopted for the studies on glucose absorption.

Everted sacs were incubated in different concentrations of C^{14} glucose and the absorption of glucose was determined as the difference between initial and final concentration of the incubation medium. Duplicate samples were analyzed by both glucose oxidase and the radiotracer methods. The results are expressed in Table I as the ratio of μg absorbed to μg in the intial solution, and CPM absorbed to CPM in the initial solution. These ratios are practically identical for each concentration indicating that the radiotracer method faithfully measures the quantity of glucose absorbed. These experiments also demonstrate that under the prescribed conditions, it is not necessary to treat the samples with TCA prior to analysis. An appreciable breakdown of glucose due to enzymes released into the medium would have caused a difference in the corresponding ratios of the two methods. The validity of the radiotracer method for all the experiments reported in this paper was established either by radiochromatography or by procedures similar to those described above.

RESULTS

Physiological medium and pH

The perivisceral fluid of holothurians is similar to the surrounding sea water in ionic composition and osmotic pressure (Farmanfarmaian, 1969). Since the animal is a particle feeder, presumably some sea water enters the digestive tract along with the food particles.

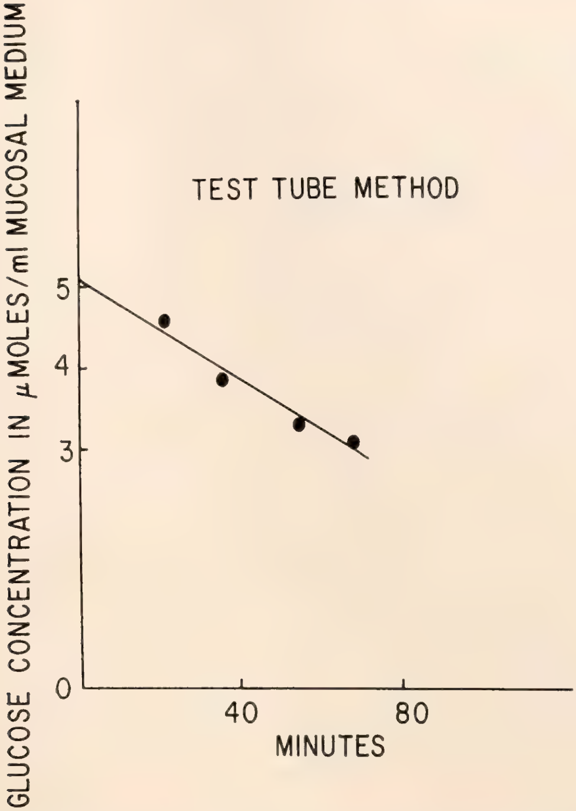

FIGURE 1. Reduction of D-glucose concentration in the mucosal medium due to absorption by the mucosal epithelium. Conditions of the experiment: Clear zone segment in test tube; mucosal medium 0.5 ml of 5 m*M* glucose FSW solution; serosal medium 4 ml of FSW.

These initial considerations prompted the use of filtered sea water as the physiological medium in which transport studies were carried out. Sea water has an appreciable amount of buffer capacity at pH 8.0 (Sverdrup, Johnson, and Fleming, 1942). Titration of filtered sea water used in the reported experiments gave a buffer capacity equivalent to 0.1 unit decrease in pH when 7 cc of N/1000 HCl was added to 100 cc of filtered sea water at pH 7.9.

In a series of experiments where 0.1–0.2 g of fresh tissue and 1.5–4 ml of unbuffered filtered sea water glucose solutions were used, the pH of the medium at the start of the experiment ranged between 7.7–8.0 and at the end of 1–2 hours incubation, 7.5–7.8. During this time glucose absorption was essentially linear (Figs. 1 and 2). These experiments showed that during long incubations the pH may drop by as much as 0.3 units but this pH change has no appreciable effect on glucose absorption in the range of 7.5–8.0. In experiments of short duration (< 20 minutes), the pH did not change. The effect of pH on glucose absorption in the range of 7.8–9.0 were studied using Tris buffered solutions of glucose in filtered sea water. The results presented in Table II show that there is a small

TABLE II

*Effect of pH on the absorption of C^{14}-glucose**

Tissue wet weight in g	pH	Initial CPM/100 μl	Final CPM/100 μl	$\frac{\text{CPM absorbed}}{\text{CPM in initial solution}}$	As per cent of pH 7.8	Per cent change
0.1837	7.8	7590	6020	0.207	100	—
	8.2	12030	9300	0.227	110	+10
	8.6	10450	8140	0.221	107	+7
	9.0	17490	13680	0.218	105	+5
0.1712	7.8	7500	6150	0.180	100	—
	8.2	12080	9510	0.213	118	+18
	8.6	10490	8260	0.212	118	+18
	9.0	17450	14020	0.196	109	+9
0.1309	7.8	7500	6260	0.165	100	—
	8.2	12080	9320	0.228	138	+38
	8.6	10490	8550	0.185	112	+12
	9.0	17450	14020	0.196	119	+19

* Conditions of experiment: First loop everted sacs in open beakers. Each sac was first incubated for 5 minutes in pH 7.8 solution, then washed in FSW and reincubated for 5 minutes in the next solution and so on. Incubation medium, 2 ml of 0.5 m*M* C^{14}-Glucose FSW buffered with 5 m*M* Tris at the given pH. Serosal medium, 0.9 ml of FSW.

stimulation of absorption at higher pH. In all cases maximum absorption was at pH 8.2. A few measurements of the pH of the intestine were made by applying the electrode to the mucosal epithelium directly. The results were between 7.6 and 8.0.

TABLE III

*Absorption of C^{14}-glucose from filtered sea water (FSW) and centrifuged perivisceral fluid (PF) solutions**

Tissue wet weight in g	Paired test solutions 1.5 ml	$\frac{\text{CPM absorbed}}{\text{CPM in initial solution}}$	Per cent difference
0.1693	0.25 m*M* glucose FSW	$\frac{5646}{8128} = 0.69$	10
	0.25 m*M* glucose PF	$\frac{2829}{4579} = 0.62$	
0.1522	0.50 m*M* glucose FSW	$\frac{2314}{8963} = 0.26$	0
	0.50 m*M* glucose PF	$\frac{25466}{97038} = 0.26$	

* Conditions of experiment: First loop everted sacs in Warburg flasks. Each sac was incubated for 10 minutes in the first solution, then washed in FSW and reincubated in the second solution for 10 minutes. pH of centrifuged perivisceral fluid was 7.5 and that of filtered sea water 8.0. Intestine and perivisceral fluid of the same animal was used. Serosal medium, 0.3 ml of FSW or PF.

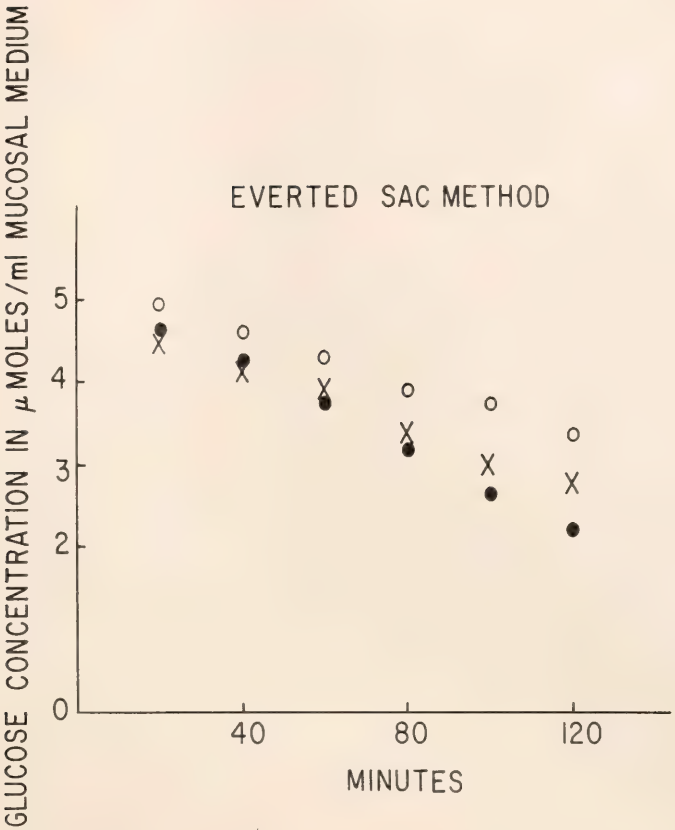

FIGURE 2. Reduction of D-glucose concentration in the mucosal medium due to absorption by the mucosal epithelium. Conditions of the experiment: Everted sacs of clear zone from three different animals; in Warburg flasks; mucosal medium 1.5 ml of 5 m*M* glucose FSW solution; serosal medium 0.6 ml FSW.

In two experiments the absorption of glucose from solutions in filtered sea water and from solutions in centrifuged perivisceral fluid was compared. The results are given in Table III. These results indicate that there is no appreciable difference between glucose absorption from filtered sea water solutions and from centrifuged perivisceral fluid solutions. Ferguson (1964) also reported no difference in glucose uptake by the digestive gland of *Asterias* when incubated in filtered sea water or centrifuged perivisceral fluid.

On the basis of the above considerations filtered sea water was chosen as the physiological medium and buffered with Tris at pH 7.9 ± 0.1 when necessary.

Net water flux

The net flux of water occurring simultaneously with glucose absorption was studied by amaranth dye dilution, using the test tube method. Samples taken at approximately 20 minute intervals through the gut cannula showed a linear glucose

TABLE IV

*Net water flux during the absorption of glucose**

Intestinal region and dry weight	Experiment duration in hours	% of total glucose absorbed	OD_{520} for Amaranth	
			Initial	Final
Clear zone 0.0155 g	1.5	80	0.32	0.31
Cloacal segment 0.0156 g	2.0	40	0.29	0.30

* Conditions of experiment: Test tube method; mucosal medium 0.5 ml of 5 mM glucose and 0.5 mM Amaranth FSW; serosal medium 4 ml of FSW. After introduction of mucosal medium, the fluid level in the lumen was raised and lowered several times in order to mix the dye solution with residual fluid. Then 100 μl of solution was removed through the glass cannula as the initial sample and similarly at the end of the experiment as the final sample. OD_{520} was determined by adding 3 ml FSW to each sample and reading against a 3.1 ml FSW blank.

absorption rate similar to that in Figure 1. There was no change in the concentration of the dye during a 1.5 and a 2 hour incubation period. The terminal data are summarized in Table IV. These results show that while 80% and 40% of the total glucose was absorbed by the mucosal epithelium of the clear zone and cloacal segments, respectively, there was no appreciable net water flux. In experiments with the everted sac method reported in Figure 2, 0.60 ml of fluid was placed within each sac by means of a syringe graduated to 0.01 cc. After a three hour incubation, the fluid content of the sac was collected on a piece of dry parafilm and measured in the same type of syringe. The final volume was within ± 0.05 ml of the original volume indicating no appreciable net water flux. Such observations have been reconfirmed in many experiments where transmural transport was measured.

Effect of captivity

The anterior parts of the digestive tract of freshly collected specimens of *Thyone* contain a large amount of particulate matter. In the laboratory the first loop and the clear zone are usually empty after a few days. It must be assumed, therefore, that the animals undergo partial starvation in captivity. For this

TABLE V

*Effect of captivity period on C^{14}-glucose absorption rate**

No. days in lab.	Absorption μmoles/min/ml tissue H_2O ± SEM
1–4	0.72 ± 0.19
12	0.38 ± 0.10
26	0.36 ± 0.08
32	0.34 ± 0.07

* Conditions of experiment: Everted sacs of the first loop; open beakers in gyrotory shaker; incubation medium 2 ml of 1 mM glucose isotonic FSW; serosal medium 0.3 ml FSW; incubation time 5 minutes; standard error of the mean (SEM) is for $n = 3$ and $P < 0.1$.

Table VI

Relative absorption of glucose by everted segments from various regions of Thyone *intestine**

Animal number	First loop	Clear zone	Second loop	Cloacal segment
1	100	100	—	15
2	100	100	28	42
3	100	110	35	63
4	100	78	—	50
5	100	106	21	14
6	100	108	44	45

* Conditions of experiments; 1.5 ml of m*M* glucose FSW in Warburg flasks; serosal medium 0.3 ml FSW; incubation time 1 hour.

reason the effect of captivity on glucose absorpion by everted sacs from the first loop was investigated. The results reported in Table V show that by the 12th day after collection, glucose absorption is reduced to 50% of the values observed for animals which had been in captivity from 1–4 days. This reduction stabilizes so that the extension of the captivity period to as long as 32 days did not cause further appreciable reduction in the capacity to absorb glucose. These data further indicate that it is best to restrict studies on transport rates to the first few days after collection when the velocities are higher. In experiments which are designed to yield comparable results, animals of similar background and preferably from the same collection should be used. These guidelines have been followed in the present studies.

Regional capacity for absorption

The different regions of the intestine of *Thyone* were anatomically defined in the previous paper. In a series of experiments, the capacity for the absorption of glucose by each region was measured and the results expressed relative to the value obtained for the first loop region (Table VI). When the different intestinal regions from the same animal are compared, the first loop and the clear zone have the highest absorptive capacities and are nearly equal in this respect. These regions appear analogous to the jejunum and the ileum of the mammals. The second loop and the cloacal segment generally show less than half the absorptive capacity of the first loop. The cloacal segment appears to have a higher capacity than the second loop in some cases, but this is probably due to greater glucose utilization; this part of the intestine is more heavily muscularized and exhibits strong tonic and rhythmic contractions when distended.

In vivo *absorption and distribution of C^{14}-glucose*

A series of experiments were carried out in order to determine the region of intestinal absorption and the distribution of labeled glucose under *in vivo* conditions. These experiments provided additional information about the relative role of the hemal sinuses and the perivisceral fluid in the distribution of the absorbed sugar (Farmanfarmaian, 1969). The procedures were as follows: The animal was relaxed, washed, dissected, and immobilized in a wax bottomed finger

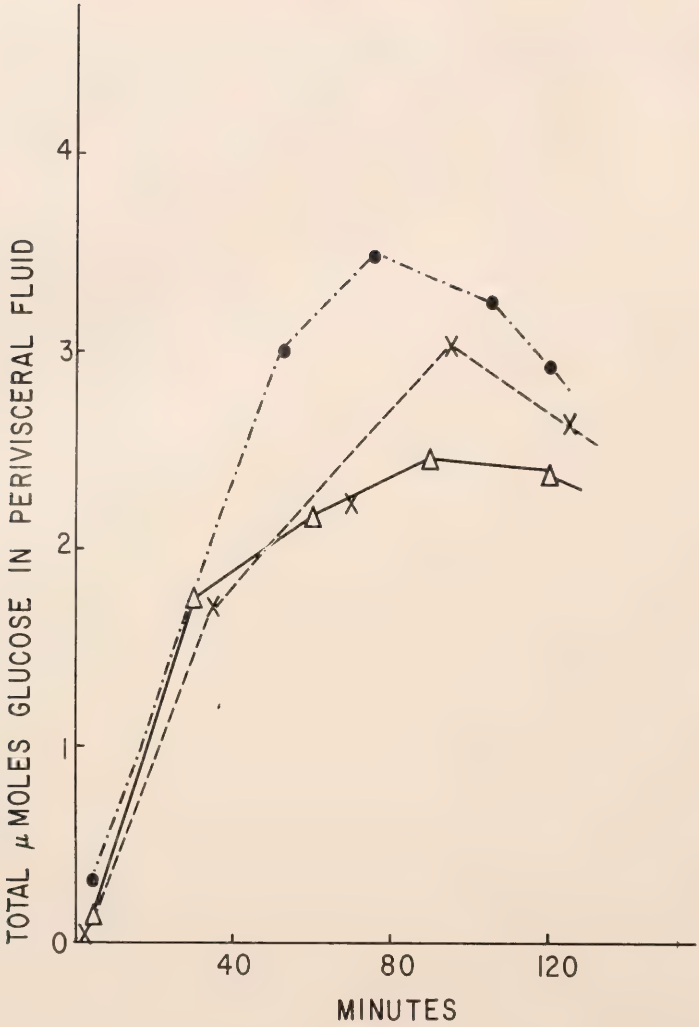

FIGURE 3. Transmural transport of D-glucose *in vivo* for three different animals. For conditions of the experiments see text.

bowl as previously described. The finger bowl was tilted and all the fluid drained. Then the animal was covered with 100 ml of filtered sea water which acts as a substitute for the perivisceral fluid. A small aerator bubbling air into the fluid, simultaneously provided aeration and mixing. The whole preparation was placed in a 20° ± 0.5° C bath and 0.5 ml of 0.5 mM glucose C^{14} and 0.5 mM amaranth in filtered sea water was intubated via the cannulated oropharynx directly into the stomach. The bathing fluid was sampled at hourly intervals. The duration of experiment was 5 hours and during this period peristalsis of the gut, circulation of the red hemocytes in the ampullae of the podia, and the movements of the branches of the water lungs were readily observable, but no circulation could be seen in the

TABLE VII

Distribution of C^{14} *activity after intubation of* C^{14}*-glucose solution into the stomach of* Thyone *under* in vivo *conditions**

Animal number		1	2
Total CPM intubated into the stomach		842250	847850
Total CPM recovered**		7575	6975
Total CPM absorbed		834675	840875
Per cent CPM absorbed		99%	99%
Total CPM in 100 ml substituted Perivisceral fluid at hourly intervals			
	hour 1	5000	3500
	hour 2	19500	25000
	hour 3	27000	28000
	hour 4	25000	21000
	hour 5	25000	30000
Per cent CPM transmural transport at the end of 3rd hour***		3.2%	3.4%
Total CPM stored in stomach and first loop tissues		807675	810875
Per cent CPM stored in stomach and first loop tissues		96.8%	96.5%
CPM/mg drained wet weight of tissue			
Stomach		85–100	150–179
Anterior first loop		816–1419	771–908
Posterior first loop		281–598	79–189
Sinuses of hemal network with contents		6	7
Clear zone		1–2	1–2
Second loop		1–2	1–2

* For conditions of the experiment see text.

** This is total activity recovered when at the end of 5 hours the posterior end of the first loop is sectioned and all the lumen fluid in the loop collected. Volume change was corrected by the Amaranth dye dilution method.

*** CPM absorbed by other tissues from the bathing fluid is not taken into account.

hemal sinuses even though these were carefully examined under the dissecting scope. The reddish-colored intubated solution reached the end of the first loop at 4.5 to 5 hours. At the end of the experiment the bathing fluid was pipeted out; the posterior terminus of the first loop was sectioned, and all the fluid from the lumen of this region of the intestine was collected. The total radioactivity in this fluid was determined after the appropriate quench corrections and the volume correction by the amaranth dye dilution method.

Most of the hemal sinuses forming the network between the first loop and the second loop were tied off in a bundle at both ends and severed beyond the ligatures. In this manner these sinuses and their contents were removed, drained of their external water on filter paper and weighed. This material was loosely spread at the bottom of a scintillation flask, Bray's scintillation fluid added, and counted. Four or more tissue samples, each measuring 0.5 cm^2, were obtained from the different regions of the digestive tract. These were similarly blotted, weighed and spread at the bottom of the scintillation flasks for counting. Tissues counted in this manner are not quench corrected and therefore provide only an estimate of the activity distribution within the tissues.

Such experiments were performed on four animals with good reproducibility. The results for two animals presented in Table VII show that 99% of the glucose was absorbed before the intubated solution reached the end of the first loop. By the end of the third hour about 3.3% of the absorbed sugar was in the substituted perivisceral fluid. The rest of the activity was stored mainly in the tissues of the first loop, particularly in the anterior part. This confirms the results of the *in vitro* experiments which showed that the first loop and clear zone are the main regions of intestinal absorption.

Although the sinuses of the hemal network are directly connected to the first loop (region of highest activity), there was no appreciable amount of radioactivity within these sinuses. By contrast, appreciable amounts of radioactivity could be recorded from the substitute perivisceral fluid at every sample interval and a peak was attained by the third hour. The magnitude of the activity in this fluid may have been 10× higher if the volume of the substitute fluid had been equal to the volume of the normal perivisceral fluid, about 10 ml. Unfortunately such an arrangement is not possible because under the experimental conditions a larger volume is necessary to cover the animal in the dish.

The amount of radioactivity in the substitute fluid was easily measurable but the concentration of glucose in 1 ml samples was below the sensitivity of the glucose oxidase method. This was due to the intubation of a meager quantity (45 μg) of high specific activity glucose so as to simulate the concentration in the natural gut fluid. In order to measure the transmural transfer of glucose by the glucose oxidase method *in vivo,* similar experiments were carried out after intubation of 2 ml of 10 mM glucose solution into the stomach and the restriction of the substitute fluid to 35 ml. One ml samples were removed at 30 minute intervals for 2 hours. These samples were directly analyzed by the purified glucose oxidase (Glucostat Special). Figure 3 illustrates the results obtained from 3 animals. These curves corroborate the isotope studies and show that glucose is rapidly transferred from the lumen to the substitute perivisceral fluid where it attains a peak in 1.5 hours. In these experiments the peak was attained in a shorter time because a larger volume (2 ml) of solution was intubated and as a result the glucose solution rapidly spread into the first loop. Of the total intubated glucose, 88–90% was absorbed by the first loop and 12–17% of the same total was found in the substitute fluid at the peak concentration.

Discussion

The studies on the effect of pH show that within the normal range of pH(s) recorded for the surrounding sea water (about 8.0), the perivisceral fluid (7.3–7.8) and the intestinal mucosal surface (7.6–8.0), change in pH has little effect upon glucose absorption by the intestinal segments of *Thyone*. At higher pH, 8.2 and above, there is an appreciable stimulation of glucose absorption. Jackson, Levin, and Thompson (1968) reported that high pH (7.8) stimulated glucose metabolism in the rat but not the mucosal transfer of glucose. It is not clear whether the stimulation of absorption noted for *Thyone* is due to increased glucose metabolism which secondarily facilitates absorption or to a direct stimulation of mucosal absorption.

Absorption of glucose was not accompanied by any appreciable absorption or transfer of water is experiments where the initial mucosal and serosal solutions were isosmotic. In the mammalian intestine, active transfer of glucose across the intestinal wall creates an osmotic gradient which results in a net transfer of water (Wilson, 1962; Smyth, 1965, 1968). In addition to this osmotic transfer, glucose and certain other sugars specificially stimulate the transfer of water *in vitro*. Barry, Smyth and Wright (1965) pointed out that fluid transfer stimulated by sugars is related to their hexokinase specificity, and therefore energy production, and not necessarily to their hexose accumulation-transport specificity as defined by Crane (1960). Glucose has both of the above specificities; therefore, in the rat intestine it is transported against a gradient and at the same time stimulates water absorpion. Galactose has only the Crane specificity and does not stimulate water absorption. Fructose has the hexokinase specificity only; it stimulates water absorption but is not transported against a gradient. Barry, Smyth and Wright (1965) concluded that fluid transfer across the intestinal wall of the rat depends on the total solute transfer. Sugars may contribute either by being a component of the solutes transferred or by providing the energy required for the transport of other solutes such as Na^+ (Schultz and Curran, 1968).

Simultaneous intestinal absorption of sugars and water have been studied in a few animals other than mammals. Fox (1961) reported that there was little water movement in either direction when intestinal segments of the turtle, *Chrysemys picta*, were incubated with different monosaccharides for one hour. Csáky and Thale (1960) and Musacchia, Neff and Westhoff (1964) reached the same conclusion for the toad, *Bufo bufo*, and the catfish, *Ictalurus nebulosus*. It is difficult to reconcile this lack of water movement with the observation of accumulation transport of sugars reported by the same authors. In the case of the bullfrog, *Rana catesbeina*, Lawrence (1963) states that net transfer of sugar is accompanied by net transfer of water.

Treherne (1967) reported that water uptake in the midgut and hindgut of several species of insects occurs both by osmosis from dilute solutions and by mechanisms linked to the active transport of Na^+. Rapid net absorption of water from amino acid solutions introduced into the midgut of the locust, *Schistocerca gregaria*, was observed by Treherne. He concluded that this was a mechanism by which the concentration of amino acids in the lumen is raised so that a downward diffusion gradient between the lumen and the haemolymph is established. Absorption of glycine and serine appears to be dependent upon the establishment of such a diffusion gradient.

For marine invertebrates, Lawrence, Lawrence, Greer and Mailman (1967) stated that there was no net movement of water, Na^+, or Cl^- across the gut of the holothurian *Stichopus parvimensis* during incubation with various sugar and amino acid solutions. This statement is in agreement with the data presented in Table IV for *Thyone*. In the amphineuran mollusk, *Cryptochiton stelleri*, studied by Lawrence and Lawrence (1967), there appears to be no net transport of water even though glucose is reported to be transported against gradient in the anterior intestine. It should be remembered that the body fluids of the three marine species mentioned above are closely similar to their environmental sea water in osmotic pressure and ionic composition. Furthermore, these species spend their entire life cycles in marine waters so that they do not normally face any water balance problems.

In summary, the above investigations of net water flux in the intestine of various animal groups indicate that absorption of water is dependent upon total solutes transfer. Its magnitude is related to the overall water balance of the animal; thus, intestinal water transport is significant for mammals and insects but not for *Thyone* or the other semiaquatic and aquatic animals cited above.

Thyone undergoes partial starvation in captivity. This results in a reduction of glucose absorption by the intestine. Similar observations were recorded from *in vivo* experiments on fasting rats when the fast period was extended to 48 hours (Wiseman, 1964). On the other hand the *in vitro* studies of Kershaw, Neame and Thompson (1960) and Crane and Mandelstam (1960) indicated that sugar absorption either did not change or was stimulated when the fasting period was extended beyond 24 hours in rats or hamsters. These investigations do not clarify the mechanisms responsible for the effects of starvation on intestinal transport mechanisms.

The combined *in vitro* investigations of the regional capacity for glucose absorption and the *in vivo* studies presented above directly support the views proposed in the previous paper (Farmanfarmaian, 1969) and permit the following conclusions for *Thyone.*

The first loop or the anterior part of the intestine is the most significant region for the absorption of sugar. The clear zone which follows the first loop provides the reserve capacity for absorption required during continuous feeding. The second loop and cloacal segment of the intestine appear to be concerned with the final formation of feces.

Depending upon the concentrations of the sugar used and the duration of the experiment, 3–17% of the absorbed sugar is directly transported into the perivisceral fluid. The hemal sinuses do not have a significant role in this translocation. The balance of the absorbed sugar is stored in the intestinal tissue, the bulk of which is represented by the large mucosal epithelial cells. It is important to emphasize that the echinoids and holothurians do not possess organs such as pancreas, liver, or hepatic caeca (Farmanfarmaian and Phillips, 1962). Therefore, digestion, absorption, and the immediate storage of nutrients is entirely the function of the mucosal epithelial cells of the gut.

Further studies directly concerned with the mechanisms of absorption and transport of sugars in *Thyone* will be published in a later communication.

I am indebted to Mr. John Krawchenko for expert technical assistance and to Dr. Parvin Saidi for her patience and moral support.

SUMMARY

In the first paper of this series the biological background for the study of absorption and transport of sugars in the intestine of *Thyone* was presented. In this paper, physiological parameters which affect the absorption of glucose such as the nature of the incubation medium, the effect of pH, the net flux of water, and the effect of starvation under captivity have been studied.

The capacity for glucose absorption by different regions of the intestine was investigated under *in vitro* and *in vivo* conditions. The first loop is the main site for the absorption and transport of sugar and the clear zone of the intestine provides the reserve capacity for absorption during continuous feeding.

The *in vivo* studies demonstrate that the intestinal tissue acts as the first storage site for the absorbed sugar. Only 3–17% of the glucose absorbed is rapidly transferred into the perivisceral fluid for distribution to other organs. The hemal sinuses are not effectively involved in the distribution of absorbed glucose. The perivisceral fluid is the functional circulatory fluid.

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CORTICAL RESPONSE ANTIGENS RELEASED AT FERTILIZATION FROM SEA URCHIN EGGS AND THEIR RELATION TO ANTIGENS OF THE JELLY COAT[1]

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The roles of specific macromolecules in the cortical response of the sea urchin egg at fertilization are complex and incompletely understood. This lack of understanding is evident when one considers the variety of functionally distinct substances which have been reported to be released during the cortical response and which may be associated with one or more types of cortical granules or cortical cytoplasm. These substances have been variously described as a sperm agglutinin ("cytofertilizin," Motomura, 1950), the precursors of the fertilization acid ("fertilization product," Ishihara, 1964, 1968ab), a membrane toughening factor ("colleterin," Motomura, 1957), a jelly precipitating factor ("antifertilizin" or JePptF (E)," (Runnström, Wicklund and Löw, 1954), a membrane elevating factor ("MbElF (E)", Runnström *et al.*, 1954), a polyspermy preventing factor (Sugiyama, 1951), the hyaline layer material (Nakano, 1956), an activating substance ("A-antigen," Perlmann and Perlmann, 1957) and an enzyme (β-1, 3-glucanase, Epel, Weaver, Muchmore and Schimke, 1969).

A necessary step toward an understanding of the functional significance of the cortical response must involve some means of making qualitative distinctions among the various macromolecules released from the egg at the time of fertilization. The present work was undertaken with the aim of using specific antibodies as analytical reagents for such a qualitative assay. The work reported here concerns the possible identity of "cytofertilizin" with fertilizin (the sperm agglutinin of egg jelly) as well as the relationship between "cytofertilizin" and the "fertilization product." Parts of this study have been reported in preliminary form elsewhere (Gregg and Metz, 1966; Metz, 1967).

Material and Methods

Spawning

The sea urchin, *Arbacia punctulata,* was used in this study. Urchins were obtained from the Marine Biological Laboratory Supply Department at Woods

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Hole, Massachusetts, or commercially from Mr. Glendle W. Noble, Florida Marine Biological Supply Company, Panama City, Florida. The animals were induced to spawn using electrical shock. Semen was shed directly into dry syracuse watch glasses and eggs were shed into filtered sea water.

Removal of egg coats

Prior to fertilization or activation, eggs were either dejellied with acidified sea water (final pH 5.0) or both the jelly and vitelline membrane were removed with trypsin. The trypsin treatment consisted of a 30 minute exposure to a 0.05% sea water solution of crude trypsin (M. B. L. stock) pH 8.2 (Tyler and Metz, 1955). Following either treatment, the eggs were washed repeatedly with filtered sea water until the supernatants no longer agglutinated sperm in a 1% suspension.

Substances released upon fertilization

Both dejellied and trypsin treated eggs were inseminated with a minimum amount of sperm sufficient to ensure at least 99% fertilization as evidenced by cleavage (final concentration approximately 5 μl undiluted semen in a 100 ml egg suspension). The concentration of the egg suspensions was about 5–10%, by volume, of freshly shed eggs. Equal volumes of eggs in all experiments were set aside as uninseminated controls. Ten minutes after insemination the supernatant sea water was decanted from the fertilized and unfertilized eggs. Sperm were removed from the former supernatant by centrifugation at 4° C for 10 minutes at 12,000 × g. Prior to further treatment, supernatants collected from both unfertilized and fertilized eggs were tested for sperm agglutinating activity. If the supernatants of the unfertilized eggs agglutinated sperm, the experiment was terminated and the solutions discarded.

Substances released upon fertilization membrane formation

In experiments involving artificially induced formation of the fertilization membrane only dejellied eggs were used. After removal of the jelly and thorough washing in sea water, these eggs were allowed to settle through millipore-filtered (0.45 μ pores) sea water into a more dense layer of an isosmotic sucrose-glycerol solution. This solution consisted of one volume of 1.08 M sucrose and four volumes of 1.08 M glycerol (Faust, Jones, and Parpart, 1959). After removal of the eggs by light centrifugation, the sucrose-glycerol solution was dialyzed against sea water (4° C) and then tested for sperm agglutinating activity. Eggs treated in this way were observed in the light microscope after removal from the sucrose-glycerol solution. They all exhibited what appeared to be a fertilization membrane. Although systematic counts were not made, a few eggs went through at least one cleavage.

Preparation of the egg jelly solutions

Solutions of egg jelly, obtained by washing unfertilized eggs in acid sea water were concentrated by precipitation from sea water with 2 volumes of cold ethanol. After at least 12 hours in the cold, the precipitate was redissolved and dialyzed against distilled water. The egg jelly used in the Ouchterlony plates contained approximately 50 μg fucose/ml according to the primary cysteine reaction (PCyRl) of Dische, Shettles and Osnos (1949).

Concentration of substances released from eggs

Following sperm agglutination tests, all of the supernatant sea water from the fertilized, unfertilized, and artificially activated egg suspensions was dialyzed against running tap water for 12 hours followed by distilled water for 24 hours, with frequent changes, at 4° C. These solutions were concentrated approximately thirtyfold in a Rinco rotary evaporator at about 30° C. Concentration of all solutions by pervaporation or lyophilyzation was equally effective. After concentration, the preparations were stored at −20° C.

Preparation of antibodies

Antibodies were prepared in 2 rabbits against the nondialyzable components present in the concentrated supernatants of dejellied, fertilized eggs. One and one-half milliters of a distilled water solution of the material (43 μg/ml of Nessler nitrogen) were emulsified with an equal volume of Freund's complete adjuvant (Difco) and administered as a single subcutaneous injection. Heart bleedings were made during the eighth week after injection. The antigenic components which were released from the eggs at fertilization were designated cortical response antigens (CRA). In addition to the anti-CRA, antibodies against other egg components were prepared in rabbits employing Freund's complete adjuvant, subscapular injections, and ear bleedings. These antibodies were prepared against dejellied unfertilized eggs (6608) and egg jelly (6612, 4/7/67; A-12, 2/20/69; A-13, 2/20/69).

Qualitative identification of antigens

The Ouchterlony method of double diffusion in agar was used for qualitative identification of antigens. The agar gel consisted of 1% agarose (Seakem, Bausch and Lomb Co.) dissolved in 0.85% sodium chloride containing 0.2% NaN_3 and 0.05% $CdCl_2$ (see Crowle, 1961). Six milliliters of melted agarose solution were pipetted onto Kodak slide cover glasses (2″ × 2″). The wells were 7 mm in diameter with a volume of about 0.1 ml and were filled only once. These Ouchterlony plates were incubated in moist chambers at room temperature for about a week. Periodic drawings were made during the incubations. When the precipitin patterns showed no further changes the plates were rinsed in running tap water for at least 24 hours prior to drying at room temperature. When dry, the plates were stained with azocarmine G (Crowle, 1961) and used as transparencies in preparing photographic records and final drawings of the precipitin patterns.

RESULTS

Release of sperm agglutinin

When a drop of a dilute sperm suspension was mixed with a drop of sea water in which dejellied or trypsin treated eggs had been fertilized the sperm immediately

TABLE I

Failure of sperm to reagglutinate after pretreatment with either fertilizin or cytofertilizin. Plus (+) and minus (−) refer, respectively, to the presence or absence of agglutination*

Solution mixed with sperm	Sperm pretreatment		
	0.05 ml of 5% sperm suspension plus 0.15 ml SW (no agglutination)	0.05 ml of 5% sperm suspension plus 0.15 ml CRA titer = 9 (agglutinated and reversed)	0.05 ml of 5% sperm suspension plus 0.15 ml EJ titer = 27 (agglutinated and reversed)
0.05 ml EJ (titer = 27)	+	−	−
0.05 ml CRA (titer = 9)	+	−	−
Sea water	−	−	−

* The egg jelly (EJ) preparation used in this experiment was prepared by diluting a pH 5.0 sea water wash of eggs. The original sperm agglutinating titer of the EJ solution obtained in this way was about 20,000. The sperm agglutinating titer of the cortical response antigen (CRA) preparation obtained upon fertilizing half of these eggs was about 9. The pH of both EJ and CRA preparations when mixed with sperm was the same pH as that of the SW used.

agglutinated. This agglutination was weak and reversed rapidly. A weak, reversible sperm agglutination was also observed when a drop of a sea water solution of the material released by the artificially activated eggs was mixed with a drop of a sperm suspension. The supernatant sea water of unfertilized dejellied control eggs did not induce sperm agglutination.

Agglutination inhibition experiments

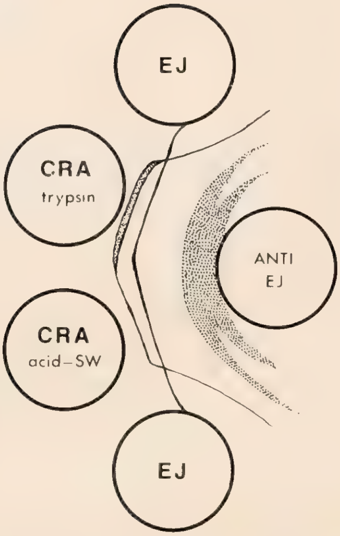
In an attempt to distinguish between fertilizin induced sperm agglutination and the agglutination induced by the supernatants of fertilized eggs, agglutination inhibition experiments were performed. Sperm suspensions were agglutinated by approximately equal concentrations of one or the other agglutinin, and the agglutination was allowed to reverse. Sperm suspensions treated in this way did not reagglutinate upon the further addition of either agglutinin. This experiment was repeated twice using different preparations with at least 5 replicate runs each time. Consistent results were obtained and the results of one such experiment are detailed in Table I.

Qualitative identification of the antigens

Whole serum from rabbits immunized against egg jelly (6612, A-12, A-13), regularly formed 4 precipitin bands when diffused in agar against concentrated egg jelly solutions. All 4 bands were continuous with 4 bands formed between antijelly and the CRA preparations from both dejellied and trypsin treated eggs as shown in Figure 1.

Of these 4 precipitin bands, 3 were observed between antijelly (6612) and the concentrated supernatants of unfertilized acid dejellied as well as trypsin treated eggs (Figure 2). In one of the concentrated supernatants of trypsin treated unfertilized eggs there was a trace of a fourth precipitin band.

1



2

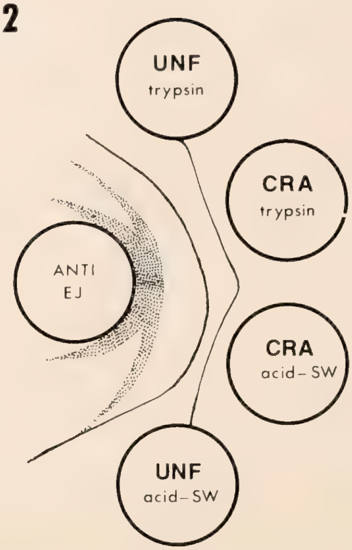


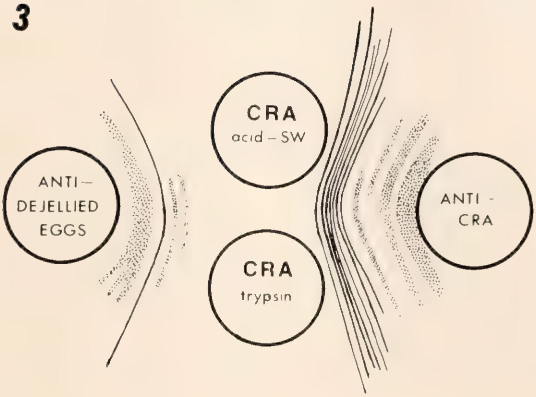
FIGURE 1. Ouchterlony agar diffusion test. The antibody well contained 0.1 ml anti-egg jelly antiserum (6612). The other wells were filled with 0.1 ml of various antigen preparations. EJ: egg jelly obtained by washing eggs with acidified sea water concentrated by alcohol precipitation. CRA trypsin: cortical response antigen preparation (concentrated 100-fold) from trypsin treated eggs. CRA acid-SW: cortical response antigen preparation (concentrated 100-fold) from eggs treated with acid sea water for removal of jelly.

FIGURE 2. Ouchterlony agar diffusion test. The antibody well contained 0.1 ml anti-egg jelly antiserum (6612). The other wells were filled with 0.1 ml of various antigen preparations. CRA trypsin: cortical response antigen preparation (concentrated 100-fold) from trypsin treated eggs. CRA acid-SW: cortical response antigen preparation (concentrated 100-fold) from eggs treated with acid sea water for removal of jelly. UNF trypsin: supernatant of unfertilized trypsin treated eggs (concentrated 100-fold). UNF acid-SW: supernatant of unfertilized eggs (concentrated 100-fold) which had been dejellied with acidified sea water.

When anti CRA (A-5) was diffused against a sample of the CRA preparation (from acid SW-dejellied eggs) which was originally injected into the rabbits, as many as 11 precipitin bands have been observed (Figs. 3 and 4). At least 10 of the 11 precipitin bands were continuous with 10 bands formed between anti-CRA (A-5) and the CRA preparations from fertilized trypsin treated eggs, as well as artificially activated eggs (Fig. 4). When the CRA preparations from acid sea water and trypsin treated eggs were diffused toward antiserum prepared against dejellied egg homogenates (6608) all the precipitin bands of the 2 antigen preparations were continuous (Fig. 4).

All of the conclusions drawn from observations of double diffusion plates were based on precipitin patterns which were duplicated at least once. The same antigen preparations from eggs of different animals yielded identical precipitin patterns. Some antigen preparations from single animals were concentrated in different ways and found to have identical precipitin patterns. However, the possibility that some precipitin bands represent artifacts introduced during concentration or immunodiffusion has not been rigorously excluded.

3



4

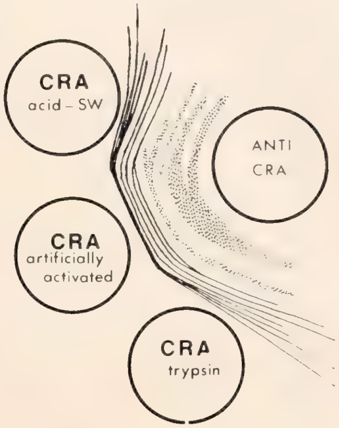


FIGURE 3. Ouchterlony agar diffusion test. Anti-dejellied eggs: antiserum (6608) prepared against homogenates of eggs which had been dejellied with acidified sea water. Anti-CRA: antiserum (A-5) prepared against a cortical response antigen preparation from eggs which had been dejellied with acidified sea water. CRA acid-SW: cortical response antigen preparation from eggs which were dejellied with acidified sea water (concentrated 60-fold). CRA trypsin: cortical response antigen preparation from trypsin treated eggs (concentrated 30-fold).

FIGURE 4. Ouchterlony agar diffusion test. Anti-CRA: antiserum (A-5) prepared against cortical response antigens from eggs which had been dejellied with acidified sea water. CRA-acid SW: cortical response antigen preparation from eggs which had been dejellied with acidified sea water (concentrated 60-fold). CRA artifically activated: cortical response antigen preparation from eggs which had been treated with a sucrose-glycerol solution as a means of artificially inducing fertilization membrane formation (approximately 30-fold concentration). CRA trypsin: cortical response antigen preparation from trypsin treated eggs (concentrated 30-fold).

DISCUSSION

Identification of cytofertilizin

Motomura (1950) introduced the term "cytofertilizin" in referring to a sperm agglutinin released from eggs of several species of Japanese sea urchins. In repeating some of Motomura's experiments, Byers (1951) failed to detect the release of a sperm agglutinin from dejellied eggs of *Arbacia punctulata.* However, the results of the present work on the same species clearly demonstrate a sperm agglutinin in the supernatant sea water of dejellied eggs following the cortical response at fertilization or artificial activation.

Similarity of cytofertilizin and fertilizin

Since both cytofertilizin and fertilizin agglutinate sperm of the species and since both reactions reverse spontaneously, the mechanisms of agglutination and the agglutinins themselves appear to be similar. This similarity of the agglutinins was confirmed in the agglutination inhibition experiments (Table I). The latter are interpreted to indicate that cytofertilizin and fertilizin combined with the same site on the sperm surface. The reversal of agglutination has been explained as a breakdown of the multivalent agglutinin into univalent fragments (Tyler, 1941; Stern, 1967).

Additional evidence for similarity of the agglutinins was obtained using immunological techniques. Since anti-jelly antiserum precipitated the agglutinin from egg jelly preparations as well as CRA preparations, it was concluded that both agglutinins were antigenic and similar. The immunodiffusion studies indicated that fertilizin and cytofertilizin were antigenically identical since all 4 egg jelly antigens were present in CRA preparations. The supernatants of unfertilized dejellied as well as trypsin treated eggs lacked one of these 4 antigens. The absence of sperm agglutinating activity in these supernatants, correlated with the absence of one antigen, suggests that this antigen is the sperm agglutinin. In the one case in which the supernatant of unfertilized trypsin treated eggs contained a trace of this fourth antigen, some cytolysis could have occurred. The trypsin treated eggs were much more fragile than acid dejellied eggs and tended to cytolyze with moderate mechanical agitation.

It has been previously reported that cytofertilizin and fertilizin were chemically and functionally distinct. Motomura (1950) inferred that the solubilities of the two substances, as a function of pH, were sufficiently different that they should be considered separate chemical entities. Hagström (1956), using other species, was not able to confirm these pH-dependent solubility relationships but he did conclude that cytofertilizin was different from fertilizin because the two had different effects on the fertilization rate.' It should be pointed out that both workers did not attempt to determine the purity of their preparations. Consequently the differing properties of their preparations of cytofertilizin and fertilizin could easily have been due to the presence of contaminating substances in either preparation. The present work indicates that there is as yet no good evidence to support the claim that cytofertilizin and fertilizin are different substances.

It is of interest that concentrated egg jelly solutions contained at least 4 antigenic macromolecules, none of which was unique to the egg jelly preparations. These results contrast with those from other species that indicate an antigenic component(s) in the egg jelly which is not found in the egg (Perlmann and Perlmann, 1957; also, see discussion, Metz, 1968).

Diffusion of an antigen from the egg surface

Assuming that the sperm agglutinin represents one antigen, the different CRA preparations contained at least 9 other antigens which precipitate with anti-CRA serum. At least 3 of these antigens were released by unfertilized, dejellied eggs. These antigens apparently diffused from the egg surface and did not require the cortical response to fertilization for release. Accordingly, these antigens were not considered to be cortical response antigens or permanent components of egg jelly. A non-permanent antigenic component of egg jelly has previously been described by Baxandall, Perlmann, and Afzelius (1964) and by Perlmann and Perlmann (1957). These antigens may also be similar to the non-agglutinating component of egg jelly to which Messina and Monroy (1956) referred.

Relationship between CRA and the fertilization product

Ishihara (1964, 1968ab) investigated the chemical nature of the organic substances in the supernatant sea water of inseminated eggs. He designated these substances the "fertilization product," assuming that they were released from the egg upon fertilization, concomitant with the appearance of the fertilization acid. Since Ishihara's chemical analyses were performed on hydrolysates of the "fertilization product," his data could not distinguish among its various macromolecular components. The results of the present work have shown that the preparation corresponding to the "fertilization product" in this species of urchin consists of at least 3 antigens released before fertilization and 7 or more antigens (CRA) released following fertilization or artificial activation. While no direct evidence has been presented, it would seem likely that some or all of the cortical response antigens are released from the cortical granules when they rupture during the cortical response.

The help of Dr. A. A. Humphries in the preparation of some of the antibodies used in this study is gratefully acknowledged.

SUMMARY

1. The supernatant sea water of inseminated dejellied or trypsin treated *Arbacia punctulata* eggs was shown to contain a sperm agglutinin which was released from the eggs after insemination. This sperm agglutinin has previously been called "cytofertilizin" in other species.

2. A sperm agglutinin was also released from dejellied eggs in which the formation of a fertilization membrane had been artificially induced.

3. Agglutination inhibition experiments suggested that the agglutinin released from dejellied fertilized eggs (cytofertilizin) and the agglutinin from egg jelly (fertilizin) bind to the same sperm receptor sites.

4. Egg jelly preparations were found to contain at least 4 antigenically distinct macromolecules. At least 1 of the egg jelly antigens was the sperm agglutinin, fertilizin.

5. On the basis of double diffusion studies using antiserum against egg jelly, the 4 antigens in egg jelly preparations were indistinguishable from 4 antigens released from dejellied eggs during the cortical response to fertilization (cortical response antigens).

6. At least 3 antigens were released from dejellied or trypsin treated unfertilized eggs. These antigens were also present in egg jelly preparations and cortical response antigen preparations, but did not induce sperm agglutination.

7. Double diffusion studies using antiserum against cortical response antigens indicated that at least 7 antigens were released from dejellied eggs when they were inseminated or when the formation of the fertilization membrane was artificially induced. The same antigens were released from trypsin treated eggs after insemination.

8. The significance of these findings is discussed in relation to cytofertilizin and the fertilization product. The results indicate that cytofertilizin and fertilizin are functionally and antigenically identical.

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PURINE EXCRETION IN FIVE SCORPIONS, A UROPYGID AND A CENTIPEDE

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Terrestrial arthropods differ in patterns of nitrogen excretion. Although insects generally are uricotelic, hypoxanthine and possibly xanthine have been identified in the moth, *Galleria mellonella* (Nation and Patton, 1961). Excreta of *G. mellonella* contained 2.6% uric acid and 0.2% hypoxanthine.

In contrast to insects, the principal nitrogenous waste of arachnids appears to be guanine. Only guanine was detected in the excreta of 34 species of spiders (Anderson, 1966). Anderson's work, in conjunction with the reports of earlier workers (Vajropala, 1935; Schmidt, Liss and Thannhauser, 1955; Atkinson and Chorlton, 1956; Haggag and Fouad, 1965), clearly illustrates the importance of guanine as the excretory product of spiders. Scorpions, however, are more inconsistent than spiders in their pattern of nitrogen excretion. Gregoire, Gregoire and Miranda (1955) reported high concentrations of guanine in the excreta of specimens of *Androctonus australis* and *A. amoreuxi*, but failed to detect either adenine or hypoxanthine. In India, Kanungo, Bohidar and Patnaik (1962) reported adenine, hypoxanthine, and uric acid, but not guanine or xanthine in excreta of *Palamnaeus bengalensis*. An extensive study by Rao and Gopalakrishnareddy (1962) showed that guanine was the most abundant purine excreted by the scorpions, *Heterometrus fulvipes, Lycas tricarinatus, Buthus tamulus*, and the uropygid, *Thelyphonus sepiaris*. Uric acid and hypoxanthine were of lesser importance. From these accounts scorpions seemingly differ both qualitatively and quantitatively in the purines excreted. Because arachnids consume considerable amounts of uric acid from their insect prey, the role of diet as an influential factor affecting purine excretion in scorpions and uropygids has been investigated. An evaluation of the malpighian tubules in purine excretion in a centipede (Chilopoda) and a scorpion also is presented.

METHODS

Excreta of the following arthropods was analyzed.

Class Arachnida:

Order Scorpiones:	Buthidae	*Centruroides marginaritatus*
	Buthidae	*Centruroides vittatus*
	Scorpionidae	*Heterometrus* sp.
	Vejovidae	*Vejovis* sp.
	Vejovidae	*Vejovis mexicanus*
Order Urogpygi:		*Mastigoproctus giganteus*

Class Chilopoda:

Order Scolopendromorpha: *Scolopendra heros*

With the exception of *C. marginaritatus* from Florida, U. S. A. and *Heterometrus* sp. from Bangkok, Thailand, all species were from Central and West Texas, U. S. A. The two undescribed species of scorpions are being described by Dr. H. Stahnke, Arizona State University. The local arthropods were maintained in the laboratory for many weeks and fed crickets (*Gryllus assimilis*). Faeces were collected for several weeks following feeding to determine if uric acid might be exogenous, and several milligrams of excreta were dissolved by heating in 0.1 N NaOH for a few minutes. The spectrophotometric method of Vischer and Chargaff (1948) and the modified phosphotungstic acid method (Eichhorn, Zelmanowski, Lewis and Fanias (1961) were used to quantitatively determine guanine and uric acid. Qualitative tests for guanine were according to Hawk, Oser and Summerson (1954). Ascending paper chromatography was used to qualitatively test for other purines. Chromatograms were developed with the mercury-diphenylcarbazone reagent of Dikstein, Bergman and Chaimovitz (1956). The solvents were as follows: (1) 95% ethanol: aqueous 0.4 N NaOH (3:1); (2) 99% isopropanol: aqueous 0.4 N NaOH (3:1); (3) isoamyl alcohol and 5% KH_2PO_4 (Carter, 1950). Total nitrogen was estimated by the microkjeldahl method (Hawk *et al.*, 1954).

TABLE I

Analysis of excreta purines of seven terrestrial arthropods

Species	% of Total excreta nitrogen	% Guanine-N of total nitrogen	% Uric acid-N of total nitrogen
Centruroides vittatus (14)*	39.1 ± 6.3	44.6 ± 14.8	0.12 ± 0.27
C. marginaritatus (1)*	—	40.0	1.8
Vejovis mexicanus (3)*	39.4 ± 3.1	91.0 ± 1.5	ND***
Vejovis sp. A** (12)*	31.9 ± 11.0	81.0 ± 20.0	0.30 (1); ND (11)
Heterometrus sp. B** (5)*	41.0 ± 10.0	63.2 ± 10.1	ND
Mastigoproctus giganteus (13)	36.7 ± 12.4	74.2 ± 27.8	1.1 (1); ND (12)
Scolopendra heros (3)	8.4 ± 0.4	ND	12.5 ± 6.0

* Number of analyses.

** Undescribed species of scorpions currently being described by Dr. H. Stahnke, Arizona State University. (A) from Jeff Davis Co., Texas; (B) from Bangkok, Thailand.

*** Not detectable.

RESULTS AND DISCUSSION

Analyses of excreta are presented in Table I. All of the arachnids excrete primarily guanine and some species inconsistently void uric acid. No other purines were found by chromatographic and spectrophotometric techniques which would have detected quantities equivalent to 5% TN. Uric acid analyses of *C. vittatus* excreta collected for a two week period following feeding are extremely variable (Table II). Most excretory samples did not contain detectable levels of

TABLE II

Percentage uric acid excreted by Centruroides vittatus *following feeding*

Number of scorpions	Time in days since feeding			
	4	8	12	16
1	0.25	1.50	4.90	0.00
1	0.20	0.50	0.00	0.00
1	0.10	1.20	2.60	0.00
2	0.00	0.00	—	—
3	0.00	0.70 ± 0.27*	—	—
6	0.00	0.00	0.00	0.00

* Mean ± Standard Deviation.

uric acid. Those individuals that do excrete some uric acid do so in quantities ranging from trace amounts to 4.9%.

Uric acid was rarely found in excreta of starved animals and was detected only once in the excreta of a vejovid scorpion and the vinegarroon, *Mastigoproctus giganteus.* In these arachnids, the role of uric acid in nitrogen excretion seems of little consequence.

Other workers have reported higher levels of uric acid in scorpion excreta. For instance, specimens of the Indian scorpions, *Heterometrus fulvipes, Lycas tricarinatus,* and *Buthus tamulus* and the African scorpion, *Buthus quinqestriatus,* excreted 8–12% and 3% of their total nitrogen as uric acid. However, all of the values of Rao and Gopalakrishnareddy (1962) on the Indian scorpions apparently are based on only one sample, except those values for *H. fulvipes* excreta where at least two samples were reported. Because of the small sample size an attempt to evaluate the physiological importance of uric acid excretion in these species is impractical. In contrast the uric acid values reported by Haggag and Fouad (1965) for *B. quinquestriatus* excreta were based on 15 specimens and therefore suggest that this buthid scorpion consistently excretes uric acid. In the present study, only the buthid scorpion excreta frequently contained uric acid, and even then usually in low concentrations. That there are both intra- and interspecific differences in the patterns of uric acid excretion even when diet is controlled suggests that the buthid scorpions truly differ from the other species in aspects of uric acid metabolism.

With the exception of *C. marginaritatus* and *C. vittatus,* guanine always accounted for more than 63% of the fecal nitrogen. Adenine and hypoxanthine were never detected. Spectral curves of excreta were almost superimposable on a standard guanine curve of similar concentration—thus illustrating the absence of interfering compounds (*e.g.,* other purines). Occasional exceptions were noted in the spectral curves of *C. vittatus* excreta where peak absorption occurred at 280 instead of 273 mμ. In these samples guanine was not quantitatively determined. Low concentrations of xanthine might account for the shift of the spectral curve, even though none was detected by paper chromatographic techniques. Similar guanine concentrations have been reported in other scorpions (Rao and Gopalakrishnareddy, 1962; Haggag and Fouad, 1965), whereas high levels of hypoxanthine were never found in the excreta of the buthid scorpion, *C. vittatus,* as it was in *Buthus tumulus* (Rao and Gopalakrishnareddy, 1962); nor was adenine detected as it was in *Palamnaeus bengalensis* (=*Heterometrus bengalensis*) (Kanungo *et al.,* 1962). In the excreta of the Indian uropygid, *T. sepiaris,* guanine accounted for 69.5% of the total nitrogen, while corresponding values for the American species, *M. giganteus* were 74.2%. Seemingly guanine is also the primary nitrogenous waste of uropygids. Uric acid was found in detectable levels in only one of twelve samples of excreta of *M. giganteus.*

Scorpions seemingly differ in patterns of purine excretion. Whether these are true differences in scorpion nitrogen metabolism or just variations resulting from dietary purines is not known. For uric acid the latter possibility seems most feasible because arachnids feed principally on uricotelic insects. This view also is supported by the many uric acid analyses of the current study which show no consistent pattern in uric acid excretion, whereas guanine is consistently excreted by all arachnids. Working with 34 species of spiders that represent 17 families, Anderson (1966) found that guanine was the only purine excreted in detectable amounts, whereas earlier workers had reported uric acid also from spider excreta (Kanungo *et al.,* 1962; Haggag and Fouad, 1965). Since only small percentages of uric acid have been reported, dietary uric acid might account for these conflicting reports. Of the total excreta nitrogen, 0.26% was attributed to uric acid in the spider, *Lycosa urbana* (Haggag and Fouad, 1965). An opposite situation was reported in piscivorous sea birds. Guanine in the bird excreta was found to be exogenous because it was only detected when the birds were fed fish (Keilin, 1958).

To determine the importance of the malpighian tubules in guanine excretion, samples from both the central tube of the midgut and hindgut of the large Thai scorpion, *Heterometrus* sp., were analyzed. By employing spectrophotometric techniques and qualitative guanine tests (Hawk *et al.,* 1954) the white material from both regions of the gut tube and from the mesenteron were identified as guanine. Such deposits are scattered throughout the mesenteron and in the diverticula leading into the central tube of the midgut. Synthesis of guanine apparently occurs in the mesenteron where excretion occurs directly into the midgut tube via the diverticula. Problems associated with transporting such an insoluble compound from the site of synthesis to that of excretion are avoided. That the transparent malpighian tubules were never noted to contain guanine crystals suggests that they are of lesser importance in guanine excretion than the mesenteron. Similar findings have been reported in some spiders (Barnes, 1968). In a few insects the gut is more important than the malpighian tubules in uric acid excretion (Wigglesworth, 1953).

Though measurable levels of uric acid have been reported in the coxal glands of the scorpions, *H. fulvipes* and *B. tamulus,* the hindgut contents contain much higher concentrations of uric acid which are of the same magnitude as those in the excreta (Rao and Gopalakrishnareddy, 1962). The coxal glands, therefore, probably are of little consequence in nitrogen excretion when compared with the mesenteron and malpighian tubules. It is possible that some of the variation in uric acid excretion might be due to irregular emptying of the coxal glands, but most of the uric acid reported from excreta comes from the gut as indicated above, and may actually be exogenous in origin.

Ample amounts of uric acid nitrogen (12%-TN) are voided with the faeces in the centipede, *Scolopendra heros.* Because centipedes consume both hard and soft body parts, the uric acid contribution to the total fecal nitrogen is low. Chitin and other undigestible nitrogenous compounds contribute more to the total nitrogen values than in arachnids which feed mainly on the easily digested soft body parts. Centipede excreta contain 8.4%-N dry wt. while arachnid excreta contain about 36%-N dry wt.

As in other mandibulates the centipede malpighian tubules are implicated as sites for uric acid excretion. Uric acid analyses of the midgut contents from just anterior to the junction of the malpighian tubules and the hindgut show uric acid levels of 0.9%, while a pool of the hindgut contents and malpighian tubules has 17% uric acid. Obviously, the centipede uric acid is not dietary and probably is excreted into the hindgut via the malpighian tubules.

From an evolutionary standpoint it is interesting that among the terrestrial arthropods the arachnids excrete guanine as their principle nitrogenous waste product whereas chilopods and insects excrete uric acid. Advantages to guanine production and excretion might be that it allows for more efficient water conservation. Guanine contains one more nitrogen atom than uric acid and is less soluble in water. Both of these properties would be of value to animals dwelling in hot, dry climates where water retention would be essential. Urea, which is highly water soluble, has not been reported in arachnids (Anderson, 1966; Rao and Gopalakrishnareddy, 1962).

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SUMMARY

1. Effects of diet on uric acid excretion of three scorpions, *Centruroides vittatus, Vejovis mexicanus* and *Vejovis* sp., and the vinegarroon, *Mastigoproctus giganteus,* have been investigated. Uric acid probably is not a normal end product of nitrogen catabolism, except possibly in *C. vittatus.* Yet even here the pattern is inconsistent. Quite likely the uric acid in arachnid excreta is exogenous in origin.

2. The most important nitrogenous waste in these arachnids was guanine. In contrast to other reports on African and Asian scorpions, uric acid and hypoxanthine are of little consequence in the North American species.

3. In the large Thai scorpion, *Heterometrus* sp., the malpighian tubules are of lesser importance in guanine excretion than the mesenteron. Apparently guanine is synthesized in the mesenteron and passes via the diverticula into the gut canal rather than being excreted by the malpighian tubules.

4. Considerable quantities of uric acid are excreted in the centipede, *Scolopendra heros,* probably via the malpighian tubules.

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OSMOREGULATION IN A MARINE CILIATE, *MIAMIENSIS AVIDUS*. II. REGULATION OF INTRACELLUAR FREE AMINO ACIDS[1]

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Some aspects of osmotic and ionic regulation in *Miamiensis avidus*, a marine ciliate, are known (Kaneshiro, Dunham and Holz, 1969). As in freshwater protozoa, the contractile vacuole is involved in regulation of cell volume. This was shown by studies on the relationship between external osmolarity and the rate of vacuolar output. Intracellular Na^+ and Cl^- are maintained lower than the external concentrations in a wide range of external salinities, but the intracellular concentrations of these ions change more or less proportionally with changes in the external concentrations. Potassium, as well as Ca^{++} and Mg^{++}, are maintained relatively constant despite large changes in the external concentrations. Although not all of the intracellular osmotic constituents were identified, the evidence suggested that *Miamiensis avidus* maintains itself hyperosmotic to the environment over a wide range of external salinities.

Information on osmoregulation in other marine protozoa is very limited. It comes primarily from studies of vacuolar function (see Kitching, 1967, for references). Measurements of the inorganic ionic composition of *Uronema filificum* have also been made (Kaneshiro *et al.*, 1969).

The concentrations of free amino acids in cells of marine invertebrates are generally large, and apparently serve an osmoregulatory function (Potts and Parry, 1964). We have analyzed the free amino acids in *Miamiensis avidus*, and have demonstrated that regulation of intracellular free amino acids is involved in osmoregulation in this marine ciliate.

MATERIALS AND METHODS

Environmental osmolarity variation and ion substitutions

Mass cultures of *Miamiensis avidus* were grown as described in Kaneshiro *et al.* (1969). Appropriate concentrations of artificial sea water (*Marine Biological Laboratory Formulae and Methods V*, 1964), were added to cell suspensions to produce high osmolarity media. Distilled water was added for low osmolarity media. In some experiments sucrose (0.72 *M*) isosmotic to 31‰ salinity sea water was

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added to maintain osmotic pressure when inorganic ion concentrations were varied. All final equilibration media, except 25% sea water, were prepared by adding equal amounts of cells suspended in the culture medium and distilled water or high osmolarity solutions. The 25% sea water test medium was prepared by adding three times the amount of distilled water to the cell suspension. In individual ion substitution experiments, Na^+, K^+ and Ca^{++} were replaced by choline$^+$, Mg^{++} was replaced by Na^+, and Cl^- was replaced by a combination of propionate$^-$ and SO_4^{--}.

Amino acid analysis

Miamiensis avidus, collected by centrifugation after the above experiments, was prepared for analysis as described by Kaneshiro *et al.* (1969). Norleucine (Technicon Chemical Co., Chauncey, New York; 2.5 μ moles/ml) was added to packed cells as an internal standard.

The intracellular free amino acids were extracted with 70% ethanol (10 ml, 5° C, 24 hr). The cell debris was then centrifuged and washed by centrifugation with 5 ml, then 2 ml, 70% ethanol. Boiling the pellet in distilled water after the ethanol extraction did not increase the yield of amino acids. The three ethanol supernatants were pooled and added to 6× their pooled volume of chloroform, and the mixture was shaken and allowed to stand for 12 hours at 5° C. The aqueous phase was removed and the chloroform phase washed with 5 ml, then 2 ml, 0.01 N HCl. The washes were pooled with the aqueous phase and the total taken to near dryness with a rotary vacuum evaporator. The sample was then redissolved in 0.01 N HCl (for thin layer chromatography and Technicon automatic amino acid analysis) or pH 2.2 Na-citrate buffer (for Beckman automatic amino acid analysis) and filtered (Millipore, 0.45 μ pore size).

Extracellular amino acids from experimental media were separated from the salts by passing a 4-ml aliquot through a column of ion retardation resin (Bio-Rad, Richmond, Calif., AG 11A8, 50–100 mesh). Distilled, deionized water was the eluant and regenerant. Ninhydrin was used for detecting amino acids and silver nitrate for detecting chloride salts in the eluate fractions.

Identification of alanine, glycine and proline was done by cochromatography with standards in two systems: 1. Two-dimensional thin layer chromatography: Eastman Chromatogram, silica gel sheet, type K 301 R2 was the support and stationary phase. n-Butanol:glacial acetic acid:water (80:20:20) and chloroform:methanol:17% ammonium hydroxide (40:40:20) were the solvent systems. Ninhydrin was the indicator. 2. Ion exchange column chromatography: Enhancement of peaks of standards on automatic amino acid analyzer recording tracings was accepted as evidence of cochromatography.

Other amino acids were provisionally identified by method 2 only.

Concentrations of individual amino acids were determined by comparisons of peak areas of amino acid analyzer recorder tracings with that of the norleucine standard. Unidentified peaks were estimated by using the leucine peak area as a reference. Values obtained for the free amino acid content of cells were corrected for inulin space of packed pellets and for cell volume changes (Kaneshiro *et al.*, 1969).

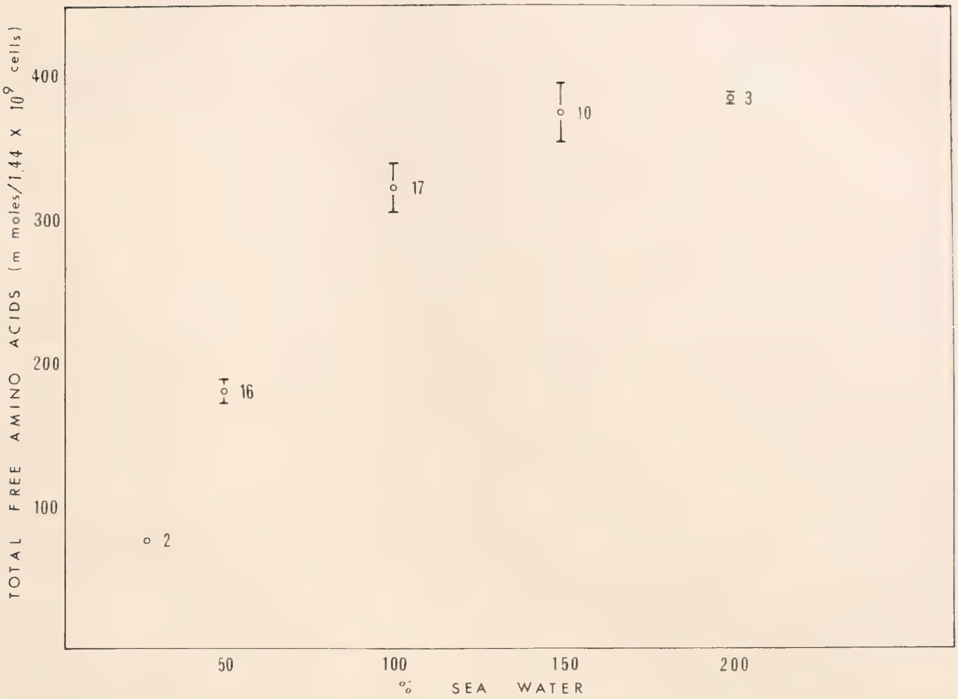

FIGURE 1. Total intracellular free amino acid content in *M. avidus* after equilibration (20–90 minutes) in media of various salinities. Since cell volumes changed under the various experimental conditions, the contents are expressed as mM/1.44 × 10^9 cells, the number of cells in 100% sea water comprising a liter. Means, standard errors of the means, and numbers of determinations are indicated.

Amino acid uptake

To determine if extracellular amino acids contributed to intracellular free amino acid pools during exposures (5–60 min) to a variety of salinities (from 25 to 200% sea water), cells were incubated with 10 mM L-alanine per liter of the various concentrations of sea water and sufficient U-C^{14}-L-alanine (Calbiochem, sp. act. —34.4 mC/mM) to give a final radioactivity of 0.02 mC/mM (4.4 × 10^5 dpm/ml) in each incubate. Radioactivities of 1-ml samples of ethanol extracts of cells, prepared as above, ethanol-insoluble cell debris (solubilized with Hyamine 10-X) and experimental test solutions were determined in a liquid scintillation spectrometer. H^3-inulin (New England Nuclear) was used for inulin space corrections (Dunham and Child, 1961).

Amino acid uptake was also tested with a mixture of U-C^{14}-L-amino acids derived from hydrolysis of a uniformly labelled algal protein (Volk Radiochemicals, Chicago, Illinois, sp. act.—0.8 mC/mg). The final radioactivity of the various concentrations of sea water was 10,000 dpm/ml. In these experiments, ethanol extracts of cells and samples of media were dried on stainless steel planchets and counted with a thin-window gas flow detector.

TABLE I

The amounts of individual free amino acids in M. avidus *equilibrated for 20–90 minutes in media of various salinities.* The amino acids are expressed as mM/1.44 × 10^9 cells, the number of cells comprising a liter in 100% sea water. The salinities are given as per cent of sea water. Numbers of determination of each amino acid at each salinity are given in parentheses

Amino acid	25(2)	50(16)	100(17)	150(10)	200(3)
Aspartic acid	0.3	1.9	1.7	4.6	1.4
Threonine	1.1	6.6	8.7	12.6	30.0
Serine + asparagine	6.4	11.8	19.3	22.6	15.6
Glutamic acid	2.8	7.7	7.8	11.0	6.1
Proline	8.1	20.9	45.6	44.9	75.3
Glycine	15.4	35.1	64.8	65.8	76.7
Alanine	27.7	66.8	128.6	154.6	142.0
Cystine	0.9	1.4	1.3	1.9	—
Valine	2.9	6.1	10.0	13.4	8.4
Methionine	0.5	0.9	1.6	2.5	0.6
Isoleucine	1.0	2.3	3.2	5.8	2.5
Leucine	1.9	4.2	6.7	10.5	4.7
Tyrosine	—	0.6	1.3	1.5	0.8
Phenylalanine	0.3	1.0	1.2	2.1	1.4
Lysine	0.9	2.6	2.2	4.6	1.2
Histidine	0.3	0.7	0.8	1.3	0.8
Arginine	1.5	2.9	3.0	3.0	3.3
Unknowns (at least 10 detected)	2.9	7.4	8.8	11.5	14.3
Total	74.9	180.9	316.6	374.2	385.1

RESULTS

The total intracellular concentration of free amino acids in *M. avidus* in 100% sea water was 317 mM/kg cells. This is in the range of concentrations in most tissues of marine metazoans which have been analyzed (Potts and Parry, 1964).

The three most prevalent free amino acids in *M. avidus* were alanine > glycine > proline. They accounted for an average of 73% of the total free amino acids in 82 determinations under various conditions.

When the external salinity was decreased, the amount of free amino acids per cell decreased and when the external salinity was increased the amount of free amino acids per cell increased. Figure 1 shows the total free amino acid content in cells equilibrated in various salinities. The effect of a two-fold reduction in salinity was much greater than the effect of a two-fold increase. Table I shows the concentrations of the individual amino acids of *M. avidus* after equilibration in the different salinities. They are listed in the order of their elution from the ion exchange column; the most acidic amino acids are retarded by the resin the least and appeared in the eluate first. Most of the free amino acids in *M. avidus* were acidic. The concentration of total free amino acids in 50, 100 and 150% sea water test solutions was 9 mM/l. The concentration in the culture medium, before dilution with distilled water or high osmolarity solution was 21 mM/l. The free amino acid concentrations of ciliates in 100% sea water culture medium and 100% sea water controls (cell suspension + 100% M. B. L. formula sea water, 1:1) were the same, so the intracellular changes were not due to changes in the

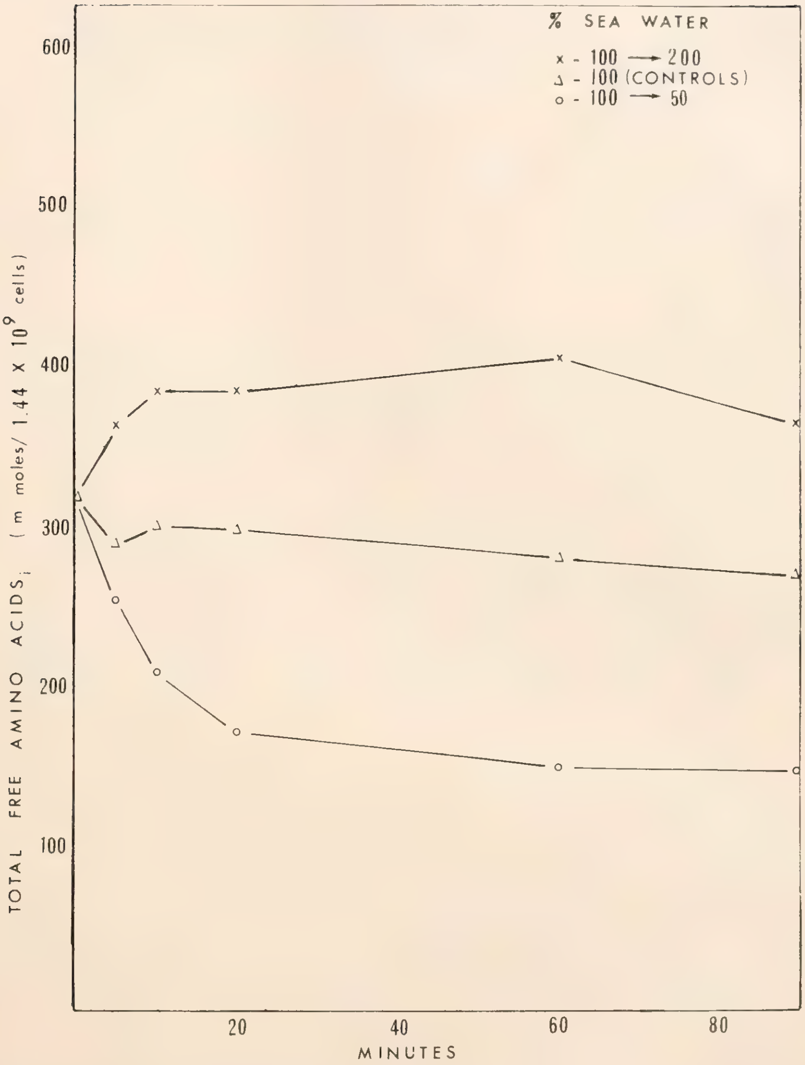

FIGURE 2. Kinetics of changes in total free amino acid content of *M. avidus* after transfer from 100% sea water to 50% or 200% sea water. Contents are expressed as mM/1.44 × 10^9 cells, the number of cells in 100% sea water comprising a liter. Each point represents a single determination.

external amino acid concentration. As shown in Figure 2, the changes in intracellular free amino acid content after transfer to either higher or lower salinity were essentially complete in 20 minutes.

Taurine, the very acidic analogue of alanine, has been detected in high concentrations in tissues of numerous marine invertebrates (Simpson, Allen and Awapara, 1959; Allen and Awapara, 1960; Awapara, 1962) but was not found in *M. avidus*.

When the external ion concentration was decreased by 50% but the external osmolarity was kept equivalent to that of sea water by addition of sucrose, the concentration of intracellular free amino acids, 285 mM/1.44 × 10^9 cells (±37 mM, S. E.; 8 determinations), was much greater than in cells in 50% sea water (181 mM/1.44 × 10^9 cells). It was not much lower than in cells in 100% sea water. Substitutions of choline$^+$ for Na^+, K^+ or Ca^{++}, Na^+ for Mg^{++} or propionate$^-$ and SO_4^{--} for Cl^- did not affect intracellular amino acid content. The decrease in intracellular free amino acid content, therefore, is a response to decreased external osmolarity and not to decreased ionic strength or specific ion concentrations.

Amino acid uptake

In none of the experiments in which cells were exposed to C^{14}-amino acids for up to one hour after transfer to higher or lower salinities was there measurable uptake of amino acids; neither C^{14}-alanine nor the mixed, labelled amino acids. In 62 samples of cells taken in 4 experiments, the radioactivities could always be accounted for by the extracellular space of the packed pellets of cells.

As shown in Table I, the measured increase of free alanine in cells after transfer from 100% to 200% sea water was 14 mM/original liter of cells. If 5% of this increase were the result of accumulation from the medium, it would have been resolved easily with the specific activity of alanine and the amounts of cells used in the experiments. Therefore, uptake of alanine from the medium could account for only a small fraction of the increase in free alanine content in the cells. A similar argument can be developed for the experiments using a mixture of C^{14}-amino acids.

Discussion

The results presented here show that osmoregulation in *Miamiensis avidus* is accomplished in part by regulation of free amino acids. Increasing or decreasing external osmolarity resulted in an increase or decrease, respectively, in intracellular free amino acids. It was shown previously that intracellular Na^+ and Cl^- are altered in a similar manner in response to changes in osmolarity. These changes, particularly of the amino acids, do not appear to be simply passive responses, but rather are likely to reflect regulatory processes enabling the cell to maintain a constant relationship between intracellular and external osmotic pressure.

It had been suggested previously, mainly on the basis of observations of the function of the contractile vacuole, that *M. avidus* maintained itself hyperosmotic to all environments, even in sea water concentrated more than two-fold (Kaneshiro *et al.*, 1969). Preliminary experiments on freezing point depressions of intact *M. avidus* cells have confirmed this suggestion.

The principal intracellular solutes which have been measured, Na^+, K^+, Cl^-, and free amino acids, total about 540 mM/kg cells in 100% sea water, and probably do not account for all the intracellular osmolarity of *M. avidus*. However, to know their actual contribution requires knowledge of cell water content, activity coefficients, and the extent and nature of compartmentation of solutes and water. The unmeasured solutes might be such substances as orthophosphate and phosphate esters, which are major constitutents in *Tetrahymena,* a fresh-water ciliate (Cline and Conner, 1966).

It is concluded that the changes in free amino acid content reflect the regulatory process which maintains the cells hyperosmotic to their environment. The functional significance of this hyperosmotic state may be to obtain water to operate the contractile vacuole. The contractile vacuole may be responsible for the elimination of some metabolic wastes. This structure may also be involved in the maintenance of the low intracellular Na^+ concentration (*cf.* Dunham, 1969). The elimination of these various solutes would require a certain volume of water, and the maintenance of a hyperosmotic state would provide the supply of water.

Maintenance of a hyperosmotic internal state is characteristic of fresh water and brackish water animals, but among true marine metazoans only the elasmobranchs are hyperosmotic. Probably all marine animals which maintain an osmolarity different from sea water are secondarily marine, and no marine animals without fresh-water or brackish water ancestors have evolved the ability to maintain an osmotic gradient. It has been postulated before that marine ciliates had fresh water ancestors (Potts and Parry, 1964), but no concrete evidence was available. Hyperosmotic regulation in marine ciliates constitutes evidence of fresh water ancestry. However, *Miamiensis avidus* is the only marine ciliate for which such evidence is available.

Decreases of intracellular free amino acid concentrations in response to salinity decreases have been observed in tissues of numerous metazoans. (*Carcinus* muscle—Shaw, 1958, Duchâteau, Florkin and Jeuniaux, 1959; *Mytilus* muscle—Potts, 1958; *Eriocheir* nerve—Schoffeniels, 1960; *Arenicola* muscle—Duchâteau-Bosson, Jeuniaux and Florkin, 1961; *Golfingia* body wall—Virkar, 1966; *Limulus* muscle—Bricteux-Gregoire, Duchâteau-Bosson, Jeuniaux and Florkin, 1966; *Crassostrea* muscle—Lynch and Wood, 1966; *Rana cancrivora* muscle—Gordon and Tucker, 1968). The decrease of free amino acid content in *M. avidus* and in the metazoan tissues in low salinities may be due to loss from the cells. However, Shaw (1958) observed no increase in the amino acid concentration in extracellular fluid of *Carcinus* when the crabs were acclimated to dilute sea water.

Prior observations of an increase in free amino acid content in response to increased salinity have been made for *Eriocheir* nerve and muscle of *Rana cancrivora*. Isolated nerves of *Eriocheir* adapted to 50% sea water increased their free amino acid concentration when placed in 100% sea water (Schoffeniels, 1960). Free amino acid concentrations in muscle of *Rana cancrivora* was much higher when the frog was acclimated in 80% sea water than when it was in fresh water (Gordon and Tucker, 1968).

The failure of *M. avidus* to accumulate C^{14}-amino acids shows that the mechanism of increased free amino acid content after increased salinity is mobilization of amino acids from protein or from other bound states within the cell. It may be surprising that no uptake of amino acids from the medium was observed under any conditions, considering that *Tetrahymena* rapidly accumulates C^{14}-phenylalanine (Stephens and Kerr, 1962).

It has been suggested that the decrease in amino acid concentration in several metazoan tissues placed in diluted sea water is due to polymerization of amino acids or binding rather than loss from the cells (Shaw, 1958; Schoffeniels, 1960; Stephens and Virkar, 1966). The decrease in free amino acids in *M. avidus* may be accomplished by the same means, the reverse of the indicated mechanism of the increase. It should be mentioned in this regard that *Tetrahymena* continually loses amino acids to an inorganic medium (Crockett, Dunham and Rasmussen, 1965; Cann, 1968).

Results presented above suggest that the changes in free amino acid content in *M. avidus* are a response to changes in osmolarity and not to changes in ionic strength, concentrations of individual inorganic ions or free amino acids. Nothing is known of the mechanism by which change in osmolarity elicits the response by the cells.

SUMMARY

1. The total free amino acid concentration in *Miamiensis avidus* in 100% sea water is 317 m*M*/kg cells. Alanine, glycine and proline account for 73% of the total.

2. Reducing the external osmolarity resulted in a decrease in intracellular free amino acids; raising the external osmolarity resulted in an increase in free amino acids. At the extreme salinities tested, cells in 25% sea water contained 24% of the free amino acids of cells in 100% sea water; cells in 200% sea water had a 22% greater free amino acid content than cells in 100% sea water. All of the changes were complete 20 minutes after the salinity changes.

3. The intracellular free amino acid content was a function of external osmolarity, and not ionic strength or concentration of individual ions.

4. The mechanism of the increase in free amino acid content was mobilization of bound amino acids, and not uptake from the medium.

5. These results, together with earlier findings on regulation of ions and water in *Miamiensis avidus*, suggest that this organism maintains itself hyperosmotic to its environment over a wide range of external salinities. The functional significance of the hyperosmotic state may be to obtain water to operate the contractile vacuole. The possibility of a fresh water ancestry for marine ciliates is discussed.

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COMPARATIVE HISTOLOGICAL STUDIES OF THE CEMENT APPARATUS OF *LEPAS ANATIFERA* AND *BALANUS TINTINNABULUM*

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Our earliest knowledge of glandular secretory structures in the Balanidae and the Lepadidae dates back to Darwin (1854) who erroneously described the unicellular cement glands as modifications of the ovarian wall. The location, function and morphology of the cement secreting glands and their associated canals were later described in more detail by Krohn (1859), Koehler (1888, 1889) Gruvel (1893, 1905, 1905a) and by Hoeck (1907), who observed structural differences in the cement complex in three genera: *Conchoderma, Lepas* and *Scalpellum.*

More recent studies on barnacles have been concerned chiefly with their taxonomy, life cycles, distribution, nutrition and ecology. However, histological investigations of the barnacle's cement apparatus have been stimulated by the current interest in natural adhesives (Lacombe, 1966, 1967). The purpose of this paper is to compare the histological characteristics of the cement glands and the conducting canals in *Lepas anatifera* and *Balanus tintinnabulum*, and to describe the pathways of glandular secretion in these representative species of the Pedunculata and Operculata.

Materials and Methods

Lepas anatifera and *Balanus tintinnabulum* were collected in Guanabara Bay, Rio de Janeiro, Brazil. The animals were fixed in Bouin-Duboscq, Bouin prepared with sea water, Carnoy, Susa, Susa prepared with sea water, formalin containing 10% calcium phosphate, Gilson and Flemming's fixatives. Decalcification was accomplished by repeated treatment with Susa's fluid. After fixation and decalcification, the tissues were dehydrated according to the following schedule which was also used for the histological preparation of excised ovaries. The tissues were dehydrated in: 95% alcohol; two transfers through 100% alcohol; 100% alcohol + benzene (3:1) for approximately four hours; 100% alcohol+ benzene (1:1) for four hours; 100% alcohol + benzene (1:3) for four hours; 100% alcohol + benzene (1:7) for six hours and benzene for ten hours before infiltration with paraffin. Tissue which was first fixed in Gilson's fluid for three hours was treated a second time in Susa's fixative followed by dehydration according to the above schedule. The same dehydration series was followed regardless of the fixative used in the study except that Bouin fixed tissue entered the dehydration series after transfer through 70% alcohol. Formalin and Flemming fixed material, after washing in running tap water for four hours, entered the dehydration series after transfers through 40% and 70% alcohol.

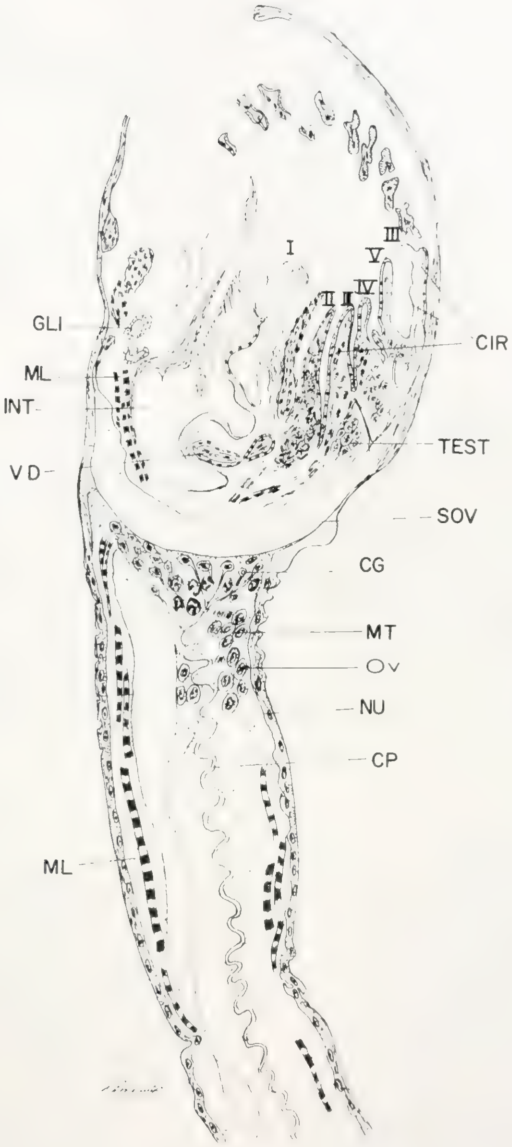


FIGURE 1. Schematic drawing of *Lepas anatifera* showing the gross morphology in cross section. CIR, thoracic appendages; TEST, testes; SOV, ovarian sac; CG, cement glands; MT, mantle tissue; Ov, ovaries; Nu, nucleus of cell in wall of peduncle; CP, principal canal; GLI, intestinal glands; ML, longitudinal muscle; INT, intestine; VD, vas deferens.

The paraffin embedded tissue was serially sectioned at 7 microns. For general histology the staining techniques of greatest value were found to be: Delafield's hematoxylin with Chromotrope 2R used as a counterstain; Ehrlich hematoxylin with

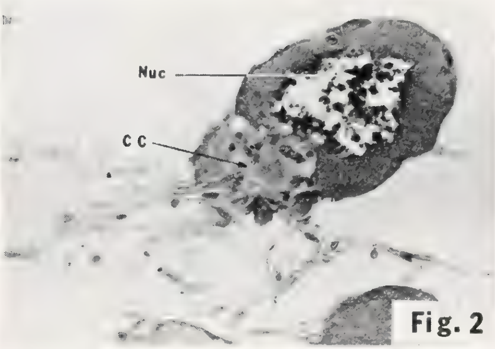
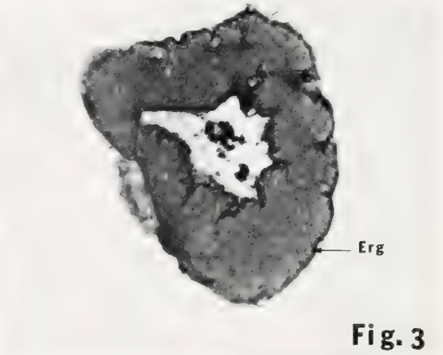


Fig. 2



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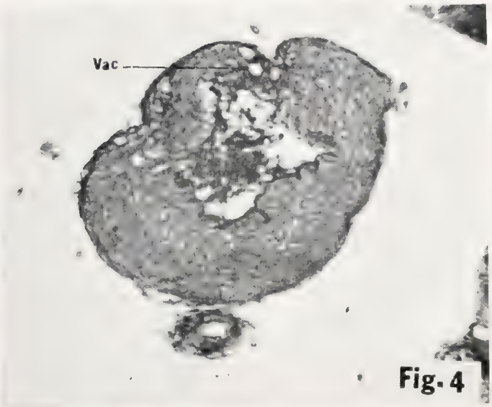


Fig. 4

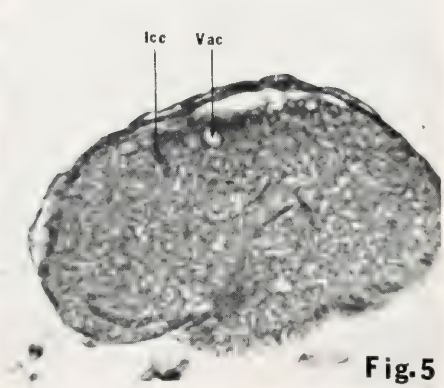
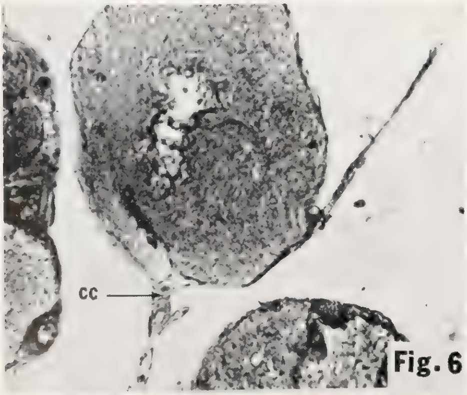


Fig. 5



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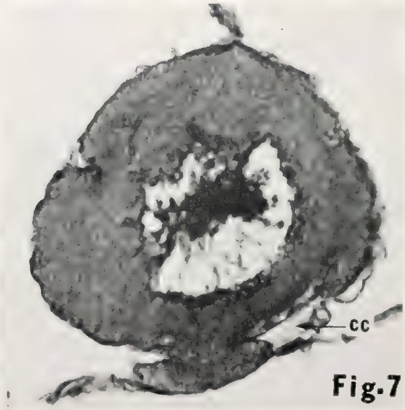

Fig. 7

FIGURE 2. Photomicrograph of unicellular cement gland of *Lepas anatifera* showing the large numbers of nucleoli (Nuc) and the collector canal (CC) at one pole of the cell.

FIGURE 3. *Lepas anatifera* cement gland showing a small zone of ergastoplasm (Erg) near the cell membrane.

FIGURE 4. Large cytoplasmic vacuoles (Vac) of *Lepas anatifera* cement gland cell.

FIGURE 5. Branches of intracellular canal (ICC) in the cytoplasm of *Lepas anatifera* cement gland.

FIGURES 6 and 7. Collector canals (CC) in the cement glands of *Lepas anatifera*, limited to a small region at one pole of the cell.

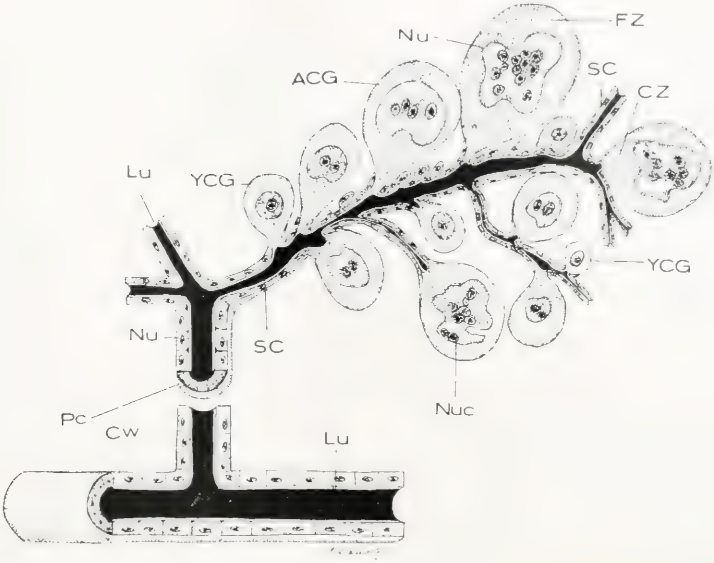

FIGURE 8. Diagrammatic representation of the cement glands and their associated canals in *Balanus tintinnabulum*. YCG, young cement gland; Nuc, nucleolus of cement gland; Lu, lumen of a cement conducting canal; CW, canal wall; PC, principal canal; Nu, nucleus; ACG, mature cement gland; FZ, formation zone for intracellular secretion; SC, secondary canal; CZ, accumulation zone for intracellular secretion.

eosin as a counterstain; nuclear fast red counterstained with naphthol green; Heidenhain's iron hematoxylin; nuclear fast red counterstained with azan and Weigert hematoxylin with Congo Red and Orange GG (1:1) used as counterstain.

RESULTS

The cement apparatus in *Lepas anatifera* and *Balanus tintinnabulum* is composed of unicellular glands and a series of connecting tubular canals which communicate from the glands to the area of barnacle attachment. Cement formed in the glands is conducted through the canals to the basal area where it hardens to form a bond with the substrate. In the present study, the terminology applied to the canals corresponds primarily to their location and function in the cement conducting system.

The principal canals are large, tubular structures which receive the cement from the smaller secondary canals. The walls of the principal canals are thick and are lined by a chitinous-like cuticle which is visible under polarized light. The principal canals of *Lepas anatifera* consist of two simple ducts that extend from the juncture they make with the secondary canals to the base by following a course parallel to the walls of the peduncle (Fig. 1).

Balanus tintinnabulum has many principal canals that conduct the cement received from the secondary canals to circular canals in the basal plate (Lacombe, 1966). In both species, the cement gland cells arise from the squamous epithelia of the walls of the secondary canals. It is assumed that the secretory products

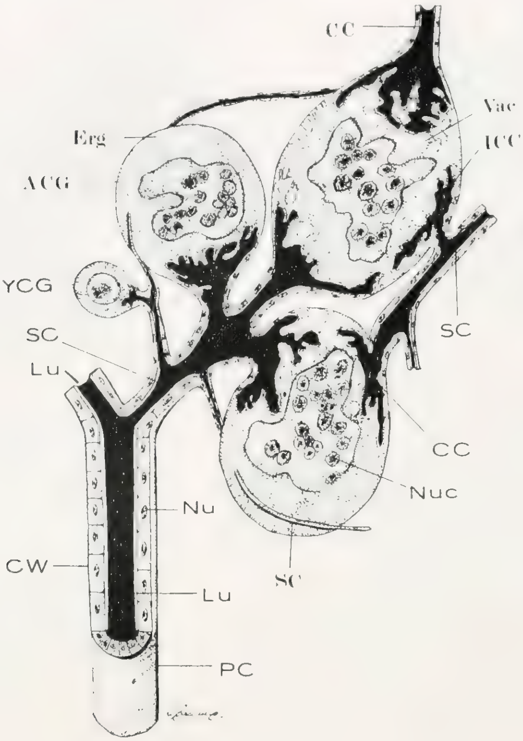

FIGURE 9. Schematic diagram of the cement glands and their canals in *Lepas anatifera*. YCG, young cement gland; ACG, mature cement gland; Erg, ergastoplasm; CC, collector canal; Vac, vacuole; ICC, intracellular canal; SC, secondary canal; Nuc, nucleolus; PC, principal canal; Lu, lumen; CW, cell wall; Nu, nucleus.

pass into the collecting canals which have direct continuity with the secondary canals from which they arise. In *Lepas anatifera* the collecting canals branch to form the intracellular canals. *Balanus tintinnabulum* lacks the intracellular canals.

The cement glands of *Lepas anatifera* (Fig. 1, CG) are found near the ovaries (Ov) in the peduncle, just beneath the capitulum or that portion of the animal which contains the major body parts enclosed in calcareous plates. These glands, measuring 30 to 40 microns in diameter in an animal 8 to 10 centimeters in length, have numerous nucleoli (Nuc) usually concentrated near the nuclear membrane and are surrounded by fine chromatin granules (Fig. 2). Each gland cell has a small zone of ergastoplasm (Erg) located near the cell membrane (Fig. 3). Vacuoles, varying in size, are found throughout the cytoplasm but in some instances the larger vacuoles (Vac) accumulate near the intracellular canals (Figs. 4 and 5, ICC). Collecting canals (CC) are limited to a small region at the cell pole nearest the secondary canal (Figs. 6 and 7). The cement glands for *Balanus tintinnabulum* and their canals are shown schematically in Fig. 8. The gland cells are dispersed among the interfollicular spaces of the ovary and are characterized histologically by a marked polarity in their staining reactions. When

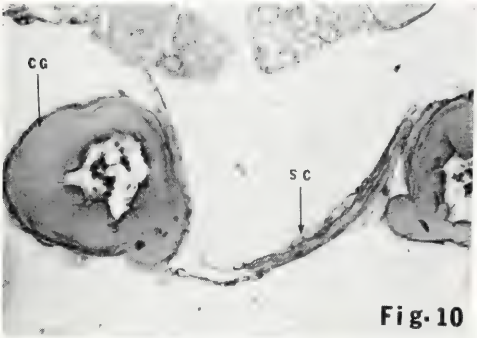

Fig. 10

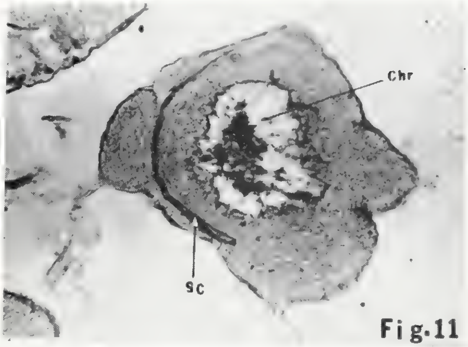

Fig. 11

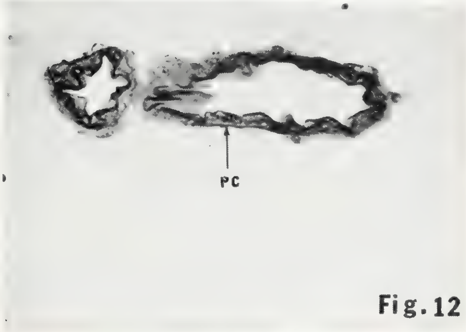

Fig. 12

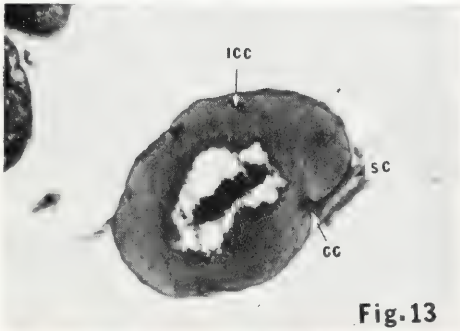

Fig. 13

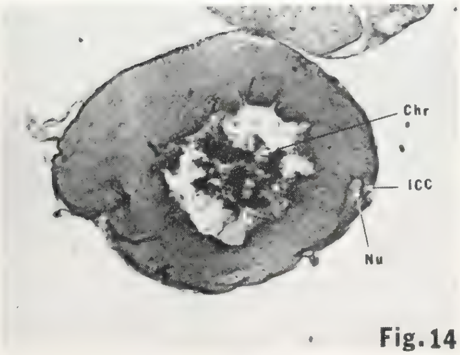

Fig. 14

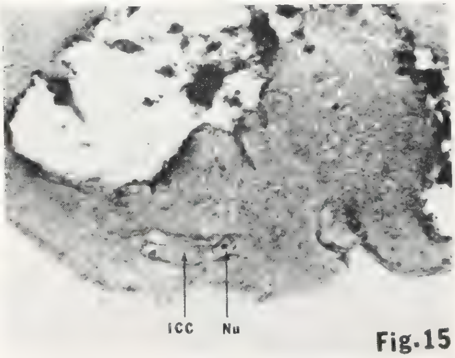

Fig. 15

FIGURE 10. SC, a secondary canal of *Lepas anatifera* shown between two cement glands (CG).

FIGURE 11. SC, a secondary canal in *L. anatifera* shown partially encircling a cement gland whose nucleus is rich in chromatin material (Chr.).

FIGURE 12. PC, a principal canal of *L. anatifera* at a point where the canal bends providing a transverse and a longitudinal view.

FIGURE 13. Canals closely associated with the cement glands of *Lepas anatifera*. ICC, intracellular canals; SC, secondary canal; CC, collector canal.

FIGURES 14 and 15. Cement gland of *Lepas anatifera*. Chr, chromatin; ICC, intracellular canals; Nu, nucleus in wall of intracellular canal.

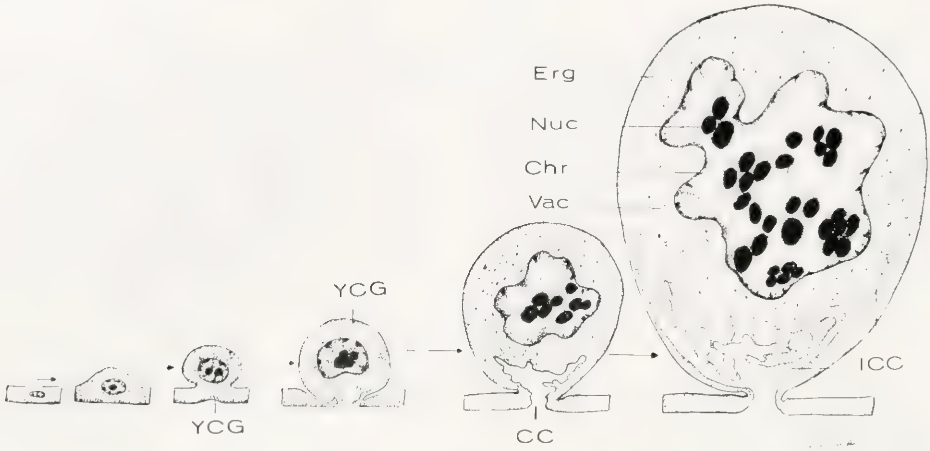

FIGURE 16. Diagrammatic representation of the development of a cement gland and its canals in *Lepas anatifera.* YCG, young cement gland; CC, collector canal; ICC, intracellular canal; Nuc, nucleolus; Chr, chromatin; Erg, ergastoplasm.

the tissue is stained with nuclear fast red using napthol green as a counterstain, the more dense zone of the cell (CZ), stains green while the less dense zone at the opposite pole (FZ), stains red. The gland cells vary in size but at maturity they may reach 40 microns in diameter. They appear singly or in groups of twenty or more cells that possess a filamentous-like cytoplasm apparently rich in mitochondria. The nucleus of a small cell (YCG) is usally round but in larger or more mature cells (ACG) the nuclei show increasing degrees of polymorphism. Chromatin is dense in the small cells but is dispersed in the large cells. Twelve or more nucleoli may be seen clearly in larger cells when fixed in Flemming's fluid and stained with Heidenhain's iron hematoxylin.

The gland cells of *Lepas anatifera*, and their association with the canal system, are shown schematically in Figure 9. Other aspects of the canal system are shown in photomicrographs in Figures 10–15. These figures show:

1. A secondary canal (SC) between two cement gland cells (CG) (Fig. 10),
2. a secondary canal partially encircling a cement gland cell which contains a large amount of chromatin (Chr), (Fig. 11),
3. the cellular structure of a principal canal (PC) in a section where the canal bends upward providing both a transverse and a longitudinal view (Fig. 12),
4. the position of a collecting canal (CC) within the cytoplasm of a cement gland (Fig. 13),
5. and the nuclei (Nu) of the walls of the intracellular canals (ICC) within the gland (Figs. 14 and 15).

The development of a cement gland (YCG), its intracellular canals (ICC) and collecting canal (CC) are diagrammatically depicted in Figure 16. A squamous epithelial cell, destined to give rise to a cement secreting cell, enlarges on the

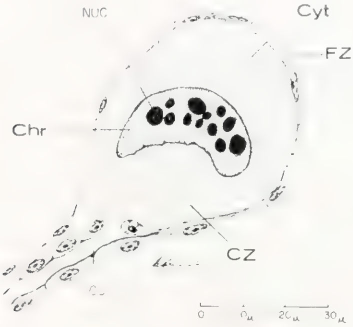

FIGURE 17. Schematic drawing of a cement gland and its canals in *Balanus tintinnabulum*. Chr, chromatin; CZ, accummulation zone for intracellular secretion; CC, collector canal; Cyt, filamentous-like cytoplasm; FZ, formation zone for intracellular secretion; Nuc, nucleolus.

wall of the secondary canal. As the cell grows the nucleus enlarges with a concomitant increase in chromatin (Chr), possibly the result of endomitosis. With continued growth, the walls of the secondary canal penetrate the cytoplasm, to form the collecting canal (CC) which branches into the tubular intracellular canals (ICC).

An individual gland cell of *Balanus tintinnabulum* is presented schematically in Figure 17 showing the two dense cytoplasmic zones (FZ and CZ), in which the cytoplasm at one pole is filamentous-like (Cyt). The other pole contains the collecting canal (CC) at the area where the glandular secretion accumulates. The nuclei, which may contain twelve or more nuceoli (Nuc), are polymorphic and have a villous surface.

An individual cement gland of *Lepas anatifera* is shown diagrammatically in Figure 18. The cytoplasm is not differentiated into two distinct zones but does contain many vacuoles (Vac). The nuclei are also polymorphic but unusually large. A fine connective tissue membrane (CT) is visible around the gland cells. Individual counts indicate the presence of more than 32 nucleoli (Nuc) of varying size surrounded by chromatin granules.

DISCUSSION

The more primitive barnacles, represented by *Lepas anatifera*, have the cement gland cells and canal system in the peduncle. In this species, cement formed in the gland cells is conducted through the primary canals to the base of the peduncle where it apparently hardens after contact with the substrate. Among the acorn barnacles, especially those members of the Balanidae with a calcareous basal plate like that of *Balanus tintinnabulum*, cement conducted through the primary canals passes into the circular canals at the base before it makes contact with the substrate.

Among closely related species of the Lepadidae, the distribution and the morphology of the cement glands and their canals may vary. Thus, Koehler (1889) reported that the cement glands of *Lepas* are limited to an area in the peduncle 2 to 3 millimeters beneath the capitulum. In *Scalpellum* the glands extend for a greater distance in the peduncle but in *Pollicepes* they are distributed

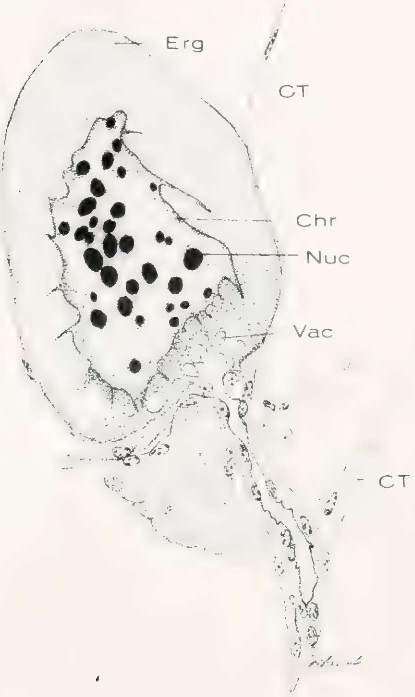

FIGURE 18. Schematic diagram of *Lepas anatifera* cement gland. Erg, ergastoplasm; CT, connective tissue membrane; Chr, chromatin; Nuc, nucleolus; Vac, vacuole.

along its entire length to the base. The cement glands of *Conchoderma*, another pedunculate barnacle, were found by Krohn (1859) to be situated in the mantle.

The distribution of the cement glands also varies with the species in the Balanidae (Lacombe and Liguori, unpublished results). For example, the cement glands of *Balanus tintinnabulum* are distributed among the interfollicular spaces of the ovaries and the connective tissue surrounding the ovarian follicles. In related species such as *Balanus nubilis*, the cement glands and their canals are not closely arranged in the interfollicular area.

Basic cytological differences in the cement gland cells of *Lepas anatifera* and *Balanus tintinnabulum*, indicate differences in the secretory mechanism. The large numbers of nucleoli, the presence of a zone of ergastoplasm and vacuoles distributed throughout the cytoplasm of the glands of *Lepas anatifera*, are characteristics of cells having a high level of metabolic activity. In this case, the glands correspond to the apocrine type in which secretory function is accompanied by a loss of some cytoplasm. The histological evidence indicates that the cellular secretion is transported to the intracellular canals in vacuoles formed in the cytoplasm. The walls of the intracellular canals, collecting canals and secondary canals have been found to be syncytial, a property which possibly enhances the transport of the soluble secretion across the cell membranes. The cement glands of *Balanus tintinnabulum* also have many nucleoli. However, they correspond to the merocrine type gland. The difference in the staining reaction at opposite poles, indicates a difference in pH. The cytoplasm is filamentous-like rather than vacuolated suggesting that the secretion formed in the zone FZ, (Fig. 17), is transported in the cytoplasm to the opposite pole CZ, where it accumulates. The change in pH between these zones may account for an increase in solubility and passage across the membrane of the collecting canal.

Examination of the histological and cytological characteristics of barnacle tissues has helped to clarify the cement forming and conducting mechanisms in these animals. Details concerning the fine structure of the cement apparatus reported by Lacombe (1968) and under investigation in this laboratory, may further clarify the mechanisms of cement secretion and conduction. It is hoped that these studies, in conjunction with histoenzymological investigations of the cement apparatus (Arvy and Lacombe, 1968; Arvy and Liguori, 1968; Arvy, Lacombe and Shimony, 1968) may lead to the chemical identification of the adhesive material secreted by barnacles.

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SUMMARY

1. The histological characteristics of the cement apparatus of barnacles have been compared in *Lepas anatifera* and *Balanus tintinnabulum*.
2. In both species the cement is formed in unicellular glands and conducted to the points of attachment through a series of tubular canals.
3. The cement gland cells of *L. anatifera* differ from those of *B. tintinnabulum* in several cytological details which may indicate differences in the secretory mechanisms.

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NERVE REGENERATION IN THE COCKROACH, *LEUCOPHAEA MADERAE:* THE EFFECT OF HUMORAL SUBSTANCES *IN VITRO*

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The process of regenerating lost appendages (epimorphosis) was divided by Needham (1952) into three stages: wound closure, demolition of damaged cells and provision for defense, and differentiation of cells to provide new tissues for the repair process. This last stage was further divided into three parts: the formation growth, and differentiation of the regenerate.

The first two stages (wound closure and demolition and defense) follow leg extirpation in immature and adult insects. These processes as they occur *in vivo* were described in detail for epidermal tissues (Wigglesworth, 1957) and nerve tissue (Bodenstein, 1957; Guthrie, 1962). However, the formation and differentiation of the regenerate leg occurs only under limited circumstances. O'Farrell and Stock (1953) demonstrated that in Blattella nymphs, regenerate formation can occur only during the earlier portion of the molting cycle. When legs are removed during the last $\frac{1}{3}$ of the cycle, wound healing occurs and a papilla-like structure is formed, but no regenerate develops until the subsequent period of intermolt. Bodenstein (1955) also pointed out that adult cockroaches that normally cannot regenerate lost appendages do so when forced to molt by the implantation of active prothoracic glands and corpora cardiaca.

Penzlin (1965) showed that development of the regenerate was closely related to the molting cycle: Development is slow when the leg is removed early in the cycle and is rapid when the leg is removed late in the cycle. A critical point is finally reached, and no development occurs until the next molting cycle. Clearly then, the development of the leg regenerate is closely linked with the molting cycle, and the process of wound healing is not. Schneiderman and Gilbert (1964) pointed out that little is known about the mechanisms that control wound healing and regenerate formation and by what means the latter is linked to the molting cycle.

Penzlin (1963) described the development of the regenerate leg of *Periplaneta* as it is formed *in vivo* and in a second paper (1964), he outlined the role of the nervous system in the formation of the regenerate. When the leg is removed at the trochanter-femoral joint, the proximal stump of the fifth mesothoracic nerve that remains in the coxa becomes the source of new neurons that migrate into the developing regenerate. As the latter develops, it grows upward to enclose the distal portion of the old nerve stump.

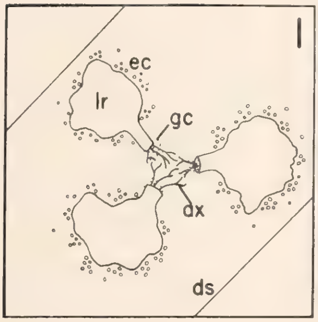
The ability to maintain cockroach leg regenerates *in vitro* was reported by Marks and Reinecke (1964). The effect of various endocrine gland explants on the development of cockroach leg regenerates was studied by the same authors (1965) who showed that the effects produced on epidermal tissues was quite different from those produced on nerve tissue. They suggested that the differences might be representative of the differences between the regenerate formation and wound healing processes and that such a system might be used to study these two processes. In a subsequent detailed study of the effects of various endocrine glands on epidermal tissues, Marks (1968) showed that these tissues did indeed respond quantitatively to prothoracic gland incubates, and this response could be modified by various *in vitro* endocrine gland interactions.

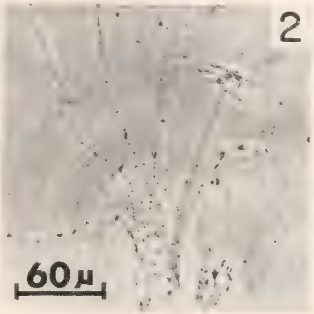
Marks, Reinecke and Leopold (1968) demonstrated that nerve regeneration *in vitro* was comparable to that reported from *in vivo* studies by Bodenstein (1957). These authors also confirmed that regenerative nerve growth does not appear to respond to the presence of the prothoracic gland in the same culture. Although these findings indicated that regenerating nerve tissue follows the wound healing pattern of growth and is controlled by a different mechanism from that which controls growth in epithelial tissues, a number of important questions remained unanswered, namely:

1. Are the effects on peripheral nerve growth produced by the presence of the ganglion in the culture unique to that organ or will nonendocrine tissues produce similar effects?
2. What effect is produced by other endocrine tissues and tissue combinations?
3. Must the endocrine tissues be present in the same chamber as the leg regenerate to produce an effect or is some kind of diffusible material produced that can give the same results as the gland itself?
4. Do interactions occur among the gland explants so that the effect produced by one gland is influenced by the presence of other glands?

Materials and Methods

A chemically defined (M-7) medium was used because it has the ability to support nerve growth in the absence of fetal calf serum (Marks and Reinecke, 1965). The preparation of the leg regenerates, ganglia, and glands was the same as that described in detail by Marks (1968). The mesothoracic legs of late instar nymphs were removed 24 hours after molting. Eight days later, the regenerates with the proximal stump of the fifth mesothoracic nerve protruding from them were dissected from the coxal stump, rinsed in nutrient medium, and placed under dialysis strips in Rose multipurpose chambers (3 or 4 to a chamber). They were arranged so that the nerve stumps were about 1 millimeter apart (Fig. 1). The endocrine glands and other tissues were removed at the same time and placed in tubes containing 1 cc of the same medium. The tubes and chambers were incubated for 6 days at 28° C. Then the chambers were checked for evidence of regenerative growth from the nerve stump with a phase contrast microscope. The emergence of glial cells from the stump of the fifth mesothoracic nerve accompanied by axons with characteristic growth cones was used as the criterion of regenerative growth since the appearance of such cells was normally followed by the linking of two explants (Marks *et al.*, 1968). If evidence of spontaneous growth was found, the leg regenerate was removed from consideration.





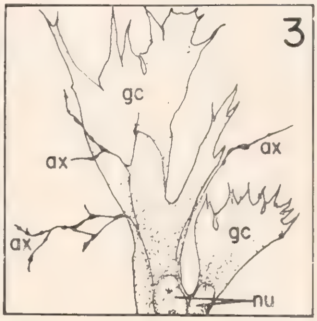


FIGURE 1. Diagram showing the arrangement of leg regenerate explants in the culture chamber. Epithelial cells (ec) surround the leg regenerate (lr) while glial cells (gc) and axon fibers (ax) emerge from the stump of the fifth mesothoracic nerve. The entire preparation is held in place by a strip of dialysis membrane (ds).

FIGURE 2. Glial cells with accompanying axon fibers emerging from the nerve stump of a cockroach leg regenerate. Dark contrast phase; 25 days *in vitro*.

FIGURE 3. Diagrammatic representation of the photograph in Figure 2. Axon fibers (ax) lying on the surface of glial cells (gc) extend out onto the surface of the cover glass. The granules that surround the nucelus (nu) probably contain glycogen.

The chambers were emptied and refilled with the medium in which the glands had been incubated, and the gland itself was discarded. After 6 more days of incubation, the chambers were again checked, and if glial cells and axons were present, the explant was scored as positive; if they were not present, it was scored as negative (Fig. 2).

Three sets of controls were used. In one set, fresh medium was added after the initial reading; the second set contained fresh medium to which was added 5% fetal calf serum; and in the third set, 1 cc of medium was added in which a 2-mm cube of cockroach muscle tissue had been incubated. The results obtained with gland incubates were tested against the controls by using the test for the difference between two sample proportions given by Goldstein (1964); significance was set at $P = 0.05$.

RESULTS

Control series

If the results of these experiments were to be meaningful, we first had to be sure that the changes in occurrence of regenerative nerve growth in the experimental chambers were not affected perceptibly by either nutrient substances or nonspecific growth factors released into the medium by the glands being tested. Therefore we ran a series of controls that incorporated three test media. The results are given in Table I.

When the chambers were refed with fresh nutrient medium, 9% of 47 regenerates showed evidence of regenerative growth from the nerve stump. When similar chambers were refed with fresh medium containing 5% fetal calf serum (a common tissue culture growth supplement), only 5% of 27 regenerates showed nerve growth. Also, when the third set of chambers was refed with nutrient

TABLE I

Control series showing the effect on nonspecific and nutrient culture medium additives on nerve regeneration in cockroach leg regenerates

Test medium	Per cent of explants showing nerve regeneration
M-7	9% of 47
M-7 + 5% fetal calf serum	5% of 27
M-7 muscle incubate (4 cu. mm muscle incubated for 6 days)	5% of 20

in which muscle tissue was incubated, only 5% of 20 showed nerve growth. These experiments indicate that the occurrence of nerve cell migration was not visibly influenced by the nutrient substances present in fetal calf serum and suggest that nerve growth is not influenced by nutritive factors supplied by the test incubates.

When the prothoracic ganglion was cultured instead of the leg regenerate, it responded to the presence of fetal calf serum with a six-fold increase in the occurrence of regenerative growth. This difference in the response of the two tissues to the presence of fetal calf serum was probably a consequence of the structure of the two organs. The leg regenerate at this stage of development is largely an epithelial sac filled with blood cells, and thus it carries a good supply of nutrient material into the culture chamber. On the other hand, the ganglion is essentially a solid organ and carries little if any blood into the culture. Thus, it is more sensitive to the presence of nutrient materials in the medium and is therefore a poor test organ for studies of this type.

It was also apparent from the control series that incubates of muscle tissue explants approximately the same size as the largest endocrine gland explants do not provide any nonspecific tissue factors that visibly effect nerve growth. Since the experimental design is such that the test tissues and the leg regenerates are never in the same culture at the same time, it can be assumed that any any influence produced by the test incubates on the leg regenerate must be caused by specific diffusible substances released by the gland explant into the culture medium during the period of incubation. It is thus possible to test the effects of incubates of various endocrine glands and gland combinations on nerve growth and to evaluate the results with some degree of confidence.

TABLE II

The effect of endocrine gland incubates on regenerative growth from the nerve stump of a cockroach leg regenerate

Tissue	Dose	Per cent of explants showing nerve regeneration
Prothoracic gland	1 gland/cc	0% of 23
	2 glands/cc	3% of 33
	3 glands/cc	0% of 21
Corpus allatum cardiacum complex	2 glands/cc	0% of 26
Brain	2 glands/cc	14% of 22
Ganglion	2 ganglion/cc	25% of 24

TABLE III

The effect of mode of incubation on the effect of incubates on nerve regeneration in the cockroach leg regenerate

Tissue	Mode of incubation	Per cent of explants showing nerve regeneration
Gland + ganglion	Separately	35% of 23
Gland + brain	Separately	14% of 22
Gland + corpora allata-cardiacum	Separately	7% of 27
Gland + muscle	Together	4% of 26
Gland + ganglion	Together	16% of 18
Gland + brain	Together	0% of 26
Gland + corpora allata-cardiacum	Together	0% of 19

Effect of gland incubates

In the first tests, incubates from individual glands were tested for their effect on the growth of nerve tissue in the regenerating leg stump. The results are given in Table II. When the incubate of prothoracic glands was tested on leg regenerates, no measurable effect on nerve cell migration was produced, regardless of the number of glands used.

Incubates of the corpus allatum-cardiacum complex also showed no measurable effect. Incubates from the brain (though they produced a frequency of nerve growth that was significantly different from that produced by the prothoracic gland and the corpus allatum-cardiacum complex) showed no significant difference from the control series. Thus, while brain incubates were not inhibitory in effect, no definite stimulation could be demonstrated. In contrast, the incubate from prothoracic ganglia had a significant stimulatory effect on the frequency of regenerative growth.

In the second series of tests, combinations of the prothoracic gland with other glands were tested. Two kinds of combinations were used: those in which the glands were combined and incubated together and those in which the glands were incubated separately and the incubates then combined. The results are given in Table III.

When the various glands were incubated separately and the incubates combined, the results were effectively the same as those obtained with the same glands without the addition of prothoracic gland incubate. However, when these same glands were incubated together with the prothoracic gland, the stimulatory effect of the prothoracic ganglion was reduced by 54%, and the borderline effect of the brain incubate disappeared entirely. The only tissues that were not affected by the presence of the prothoracic gland during the incubation process were the corpus allatum-cardiacum complex and the muscle. Since the two series differed only in the mode of incubation of the glands, the test in which the glands were incubated separately served as a control for the second set of tests; as an additional control, a prothoracic gland was incubated with a piece of muscle tissue. When the incubate was tested, the results were the same as when these same tissues were tested separately (no evidence of interaction was evident during the incubation period).

Discussion

The migration of axons and glial cells from the nerve stump of the leg regenerate was related to nerve regeneration *in vitro* by Marks *et al.* (1968). Marks and Reinecke (1965) demonstrated that the presence of the prothoracic ganglion in the same chamber stimulated this cell migration. Our present experiments demonstrate conclusively that this stimulatory effect can be produced by a diffusible substance that is carried from one culture to another in the nutrient medium. The possible presence of a nerve growth stimulator in brain incubates as well suggests that the substance may be common to all ganglionic tissues in the central nervous system. Failure of either the prothoracic gland or the corpus allatum-cardiacum complex to produce an effect further suggests that the stimulation is probably nonendocrine. The nature of this substance is unknown. However, a nerve growth stimulator isolated from vertebrate sources was identified as a protein by Levi-Montalcini (1964).

In an earlier study of leg regenerate growth, Marks (1968) demonstrated that high titers of prothoracic gland secretion produced an inhibitory effect on regenerative processes in epidermal tissues. Similarly, O'Farrell and Stock (1953) and Penzlin (1965) demonstrated *in vivo* that regenerate formation did not occur during the latter part of the intermolt when the effect of molting hormone is presumably maximal. It is of particular interest, then, to know what effect the prothoracic gland has on the regenerative growth of nerve tissue.

When the brain, prothoracic ganglion, and allatum-cardiacum complex were incubated separately and the incubates combined with incubates of a single prothoracic gland, the failure of the various incubate combinations to produce an effect different from that produced by these same glands alone suggests that the prothoracic gland itself produced little or no effect on nerve growth *per se*. In addition to this series that served as a control for the next series of experiments, the gland-muscle combination gave similar results that eliminated the likelihood that nonspecific tissue factors might affect the interaction among the glands.

When the ganglion and prothoracic glands were incubated together and the resulting incubate was tested, we found a sharp reduction in the frequency of nerve growth. When compared with the preceding experiment, the reduction clearly indicated that an interaction had occurred between the glands during the period of incubation. The result was a decrease in the frequency of regenerative nerve growth.

There are two possible explanations for these results: The first derives from the fact that the ganglion was demonstrated to stimulate the prothoracic gland *in vitro* (Marks, 1968). Thus, the increased titer of secretion from the prothoracic gland might inhibit nerve growth *per se* by acting directly on the nerve tissue. However, when the same glands were incubated separately, the frequency of regenerative activity produced was not decreased by the presence of prothoracic gland incubate. This hypothesis must thus be discarded.

The second explanation proposes that the prothoracic gland acts on the ganglion to inhibit its production of nerve growth factor and that it produces little or no direct effect on the nerve itself. This hypothesis not only accounts for the apparent lack of activity of the prothoracic gland secretion on the regenerate but it also explains why the brain and ganglion produced similar effects when allowed to interact with the prothoracic gland. This explanation appears to be the correct one. The production of nerve growth factor by the ganglion is thus regulated, at least in part, by a diffusible substance secreted by the prothoracic gland.

Three conclusions can be drawn from these findings: (1) A diffusible substance is released by the prothoracic ganglion when it is incubated in a chemically defined nutrient medium. This substance increases the frequency of regenerative nerve growth in cockroach leg regenerates *in vitro*. (2) The prothoracic glands, the allatum-cardiacum complex, and the muscle tissue showed no such activity. (3) When the ganglion and prothoracic gland were allowed to interact, the stimulatory effect was reduced by more than 50%. The prothoracic gland apparently depressed the production by the ganglion of the growth-stimulating material. Assuming that these *in vitro* findings are representative of the process of nerve regeneration as it occurs *in vivo,* they can be related to the overall process of leg regeneration. Marks (1968) showed that a high titer of prothoracic gland secretion similar to that present during the last part of the molting cycle inhibits the development of a leg regenerate by acting on the epidermal tissues. Penzlin (1964) demonstrated that the development of the musculature of a leg regenerate is partially dependent on the previous regeneration of the nerve. Thus, the high level of prothoracic gland secretion that inhibits the development of epidermal tissues also retards the regeneration of nerve and indirectly of muscle tissue by inhibiting the production of nerve growth factor by the ganglion. It is scarcely surprising that under these conditions, a leg regenerate fails to develop until the following instar.

SUMMARY

An *in vitro* system was used to study the effects of diffusible substances from various endocrine tissues on the growth of nerve tissue from regenerating cockroach legs. Incubates prepared from endocrine glands and gland combinations were tested for their effect on regenerative growth from the stump of the fifth mesothoracic nerve of 8-day leg regenerates from late instar nymphs of *Leucophaea maderae*. Incubates prepared from the prothoracic ganglion produced a stimulatory effect, while the prothoracic gland, allatum-cardiacum complex, and brain showed no effect. When prothoracic gland and prothoracic ganglion incubates were combined, the results were the same as with ganglion incubate alone. When the glands were incubated together and allowed to interact, the nerve growth stimulating effect of the ganglion was reduced by 50%. To explain these findings, it is postulated that a diffusible substance is released by the prothoracic ganglion which stimulates regenerative nerve growth from the leg regenerate. The release of this nerve growth stimulating factor is inhibited by interaction with the prothoracic gland. The possible effect of this interaction on the process of leg regeneration as it occurs *in vivo* is discussed.

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LIFETIME REPRODUCTIVE PERFORMANCE OF MICE EXPOSED AS EMBRYOS TO X-IRRADIATION[1]

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In previous studies by Nash and Gowen (1962, 1965) it was shown with mice that following X-irradiation *in utero* postnatal growth and lifespan were dependent on both the amount of irradiation and the embryological age at the time of irradiation. In addition to the above characteristics, lifetime fecundity and fertility have also been studied in the same mice to evaluate further the long-term effects of embryonic and fetal irradiation. The present paper deals with those changes induced in the developing reproductive system as reflected in total lifetime reproductive performance.

In addition, examination of important genotypic-environmental interactions was investigated by studying the reproductive responses of several genetic backgrounds through the use of inbred strains of mice and the hybrids derived from crossing them.

Exposure of the mammalian embryo to ionizing radiations exposes rapidly dividing cells during periods of organ formation and differentiation. Many studies on the effects of acute irradiation on mammalian prenatal development have established that the type of developmental malformation is dependent on both the level of irradiation and the embryological stage at the time of treatment (reviews by Russell, 1954; O'Brien, 1956). The great majority of these studies has been concerned with those changes that have been evident within a short time of irradiation. Investigations of the long-term sequelae of embryonic or fetal irradiation have been somewhat limited.

MATERIALS AND METHOD

Three inbred strains of mice and the six possible types of hybrids derived from them were utilized in these investigations. The Committee on Mouse Nomenclature has designated the strains as BALB/Gw, K, and S. The three strains were chosen from those that were available at the Genetics Laboratory of Iowa State University at the time and represent a wide range of the spectrum of radiation response, the BALB and K strains representing relatively susceptible strains and the S strain representing a relatively resistant strain. The strains were differentiated originally by resistance to mouse typhoid (Gowen, 1948), but are known to differ in a number of other physiological characteristics including differences in response to various effects of irradiation (Grahn, 1954a, 1954b; Gowen and Stadler, 1956; Nash and Gowen, 1962, 1965; Stadler and Gowen, 1957a, 1957b).

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Matings within and among the three inbred strains of mice were set up. Females were examined daily for the presence of a vaginal plug, the plug being the sole criterion used to time the period of gestation and the approximate age of the embryos at the time of irradiation. The time of 4:00 AM was used as an approximate time of fertilization since it has been demonstrated by Snell (1940) that in the Bagg strain of mice, at least, fertilization occurred shortly after liberation of the egg which took place most often between midnight and 3:00 AM. Thus, the afternoon of the day on which a plug was observed was considered to be day $\frac{1}{2}$.

Pregnant females were exposed to a single whole-body dose of X-rays on day $6\frac{1}{2}$, $10\frac{1}{2}$, $14\frac{1}{2}$, or $17\frac{1}{2}$. Only first litters were used throughout these studies. The source of irradiation was a General Electric Maxitron which operated at 250 pkv, 30 ma with 0.25 mm Cu + 1 mm Al filtration at a distance of 50 cm from anode to mid-mouse. The dose rate was approximately 133 r/minute, the dosage rates having been measured in air by means of a rate meter. Pregnant mice were exposed within a circular, wooden container, $6\frac{1}{2}$ inches in diameter, and 1 inch in depth. The base of the container was $\frac{1}{4}$-by $\frac{1}{4}$-inch wire mesh, and the top was covered with two layers of cellophane. Four levels of irradiation were employed: 20, 80, 160, and 320 roentgens as well as nonirradiated controls.

Litters were recorded within 12 hours of birth and postnatal growth followed through 75 days of age (Nash and Gowen, 1962). All litters were weaned at 30 days, and the males and females separated at this time. At 75 days of age mice, irradiated or nonirradiated, were individually mated to nonirradiated mice of the Z strain. Previously unmated Z males or females were used in these matings. The treated animals were kept mated to Z mates for the remainder of their lives. Within each radiation-dose–embryological-age treatment, a total of two males and two females of each inheritance type was sought. It was not possible to have a completely orthogonal design due to mortality within some of the experimental groupings. For example, of the mice treated with 320 r at $6\frac{1}{2}$ days, or with 160 r and 320 r at $10\frac{1}{2}$, none survived until maturity. When a Z mate died before its treated partner, it was replaced within a few days by another virgin Z mouse of an age between two and six months.

All matings were observed daily and the following information taken: (a) total number of litters, (b) number of progeny within litters, (c) birth weights of individuals, (d) viability of mice within litters from birth to 21 days. Females were allowed to raise their litters, and the males were in the cages continually so that post-partum matings were possible. The mice were maintained in a well ventilated room, in which the environment was relatively constant. Food and water were available *ad libitum*. Additional data from these experiments describing effects on growth and life-span are provided in several papers including Nash and Gowen (1961, 1962, 1963a, 1963b, 1965).

RESULTS

Of the various treatment combinations, only irradiation at day $10\frac{1}{2}$ with a dose of 80 r and above produced any gross congenital malformations. The types of malformations were similar to those reported in the mouse by other workers. None of the mice that were grossly malformed survived beyond the first day of

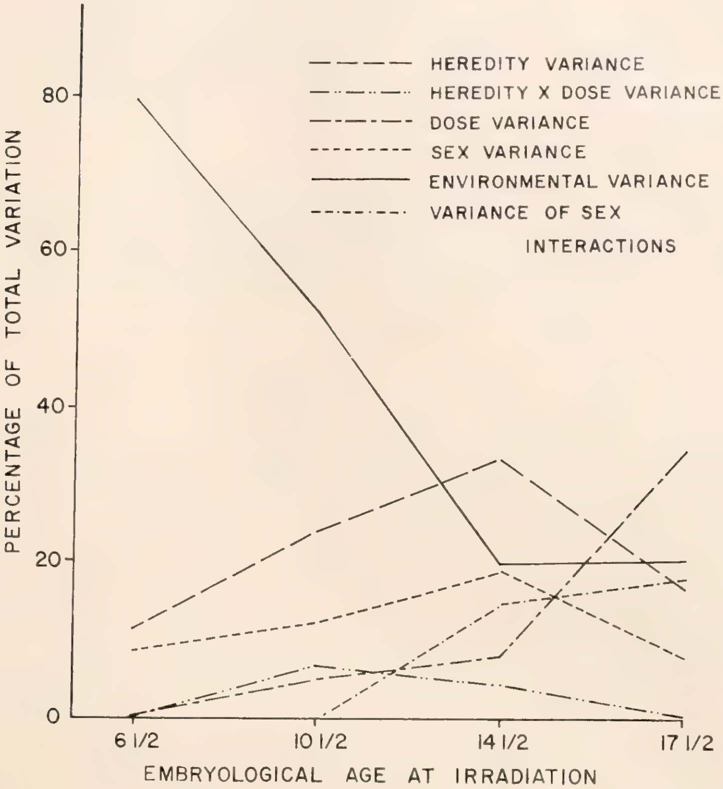

FIGURE 1. Contributions to variance of lifetime production of numbers of litters following in utero exposures to specific x-ray dosages received at different stages in embryological development.

life. Postnatal survival also was observed to be dependent on the level of irradiation and the embryological age at the time of irradiation. The mice that were subsequently tested for reproductive performance thus represent a sample of mice that had exhibited no congenital malformations and had survived to 75 days of age.

The number of sterile matings and the mean number of litters and progeny produced by males and females are shown in Table I. The overall incidence of sterile matings was low being pronounced only following irradiation at 17½ days with 160 r and 320 r, which produced 14% and 67% sterile matings, respectively. Following these two treatments 47% of the males were sterile and 33% of the females.

For both number of litters and number of progeny, similar analyses have been made. It is possible to arrange portions of the data to retain the statistical principle of orthogonality, but it does require a series of separate analyses. This has been accomplished by doing a separate analysis of variance for each of the embryological ages and for each of the levels of irradiation (Tables II and III).

Examination of the means for number of litters and number of progeny reveals that adult reproductive performance following embryonic irradiation is related both to the age of the embryo when irradiated and the level of irradiation.

Irradiation at 6½ days

Irradiation at 6½ days with doses up to 160 r apparently had little effect on subsequent fertility. Lack of effect on embryos that have been irradiated at this age but survive to be born have previously been noted for growth (Nash and Gowen, 1962) and lifespan (Nash and Gowen, 1965). Analysis of variance of these data revealed that there were significant differences due to genotype and sex, but not due to level of irradiation.

Irradiation at 10½ days

Embryos that had been exposed to 160 r or 320 r were stillborn or died within a few days of birth, so that the dose of 80 r was the highest dose tested in the reproductive studies. Although 80 r reduced the number of litters and progeny, the difference was not significant. Strain and sex effects were observed to be highly significant.

Irradiation at 14½ days

Only three embryos survived to maturity after 320 r and all were sterile. In addition to significant strain and sex effects, a significant treatment effect also

TABLE I

Incidence of sterility and mean number of litters and progeny produced in lifetime matings

Embryological age at irradiation	Irradiation dose	Females			Males		
		Number sterile	Mean number litters	Mean number of progeny	Number sterile	Mean number litters	Mean number of progeny
Control	0 r	2	9.1	79	0	12.3	94
6½ days	20 r	0	8.8	78	0	12.1	98
	80 r	0	7.7	68	0	12.0	93
	160 r	0	10.2	79	0	11.4	81
10½ days	20 r	0	8.0	77	0	12.5	94
	80 r	0	6.9	56	0	10.2	76
14½ days	20 r	0	9.3	74	0	12.8	95
	80 r	1	7.5	71	1	13.3	96
	160 r	3	6.1	45	0	10.8	80
17½ days	20 r	1	8.9	73	0	14.4	94
	80 r	0	7.6	62	0	13.2	97
	160 r	1	6.5	48	4	14.1	96
	320 r	11	2.9	24	13	2.3	12

TABLE II

Analysis of variance of number of litters produced in lifetime matings

Source of variation	Embryological age in days at exposure to irradiation							
	6½		10½		14½		17½	
	df	M.S.	df	M.S.	df	M.S.	df	M.S.
Dose (T)	3	6	2	33	3	67**	4	520**
Strain (G)	8	61*	8	71**	8	143**	8	138**
T × G	24	22	16	25	24	16	32	26
Sex (F)	1	380**	1	436**	1	803**	1	580**
T × F	3	19	2	11	3	10	4	89**
G × F	8	26	8	18	8	16	8	57**
G × T × F	24	22	16	10	24	18	32	24
Unaccounted for (E)	72	23	54	23	72	15	90	18

* 5% level of significance.
** 1% level of significance.

TABLE III

Analysis of variance of number of progeny produced in lifetime matings

Source of variation	Embryological age in days at exposure to irradiation							
	6½		10½		14½		17½	
	df	M.S.	df	M.S.	df	M.S.	df	M.S.
Dose (T)	3	531	2	3,851	3	5,545**	4	34,393**
Strain (G)	8	4,772*	8	5,454**	8	10,132**	8	10,224**
T × G	24	1,397	16	1,820	24	1,014	32	1,191
Sex (F)	1	11,025*	1	10,760**	1	25,229**	1	20,416**
T × F	3	1,028	2	124	3	1,158	4	2,881*
G × F	8	1,846	8	1,238	8	1,048	8	2,165
G × T × F	24	1,300	16	354	24	1,076	32	1,692
Unaccounted for (E)	72	2,250	54	1,448	72	694	90	1,137

* 5% level of significance.
** 1% level of significance.

was observed. Although exposure to doses through 80 r did not appear to affect reproduction, the dose of 160 r reduced number of litters to 79% and number of progeny to 72% of control values.

Irradiation at 17½ days

Sufficient progeny survived all levels of irradiation at 17½ days to enable all experimental subclasses to be tested for reproductive performance. Among the females, there was a decrease in the number of litters and progeny following all levels of irradiation. Among the males, fertility appeared normal through doses including 160 r, but dropped markedly after 320 r. The analysis of variance

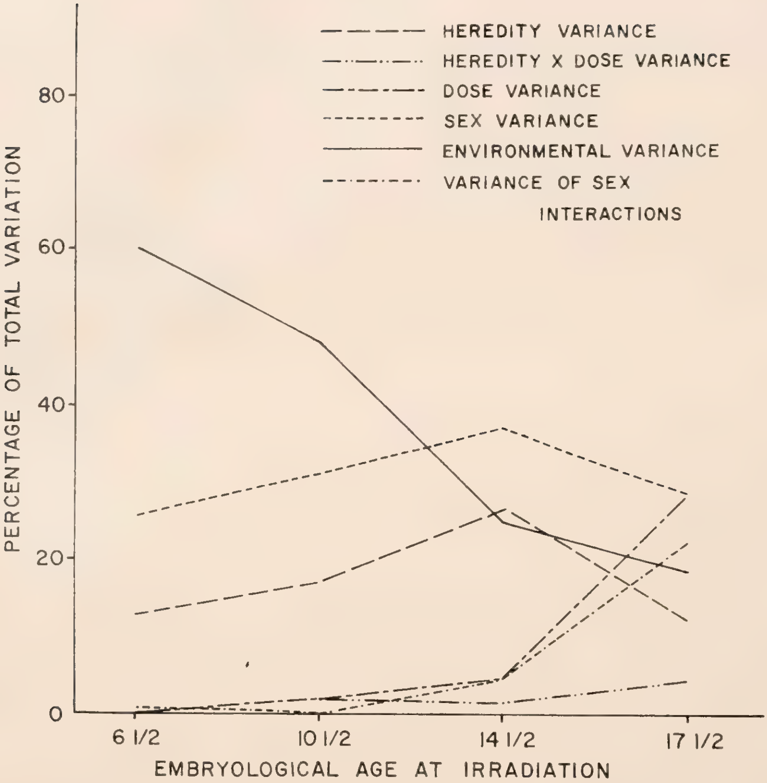

FIGURE 2. Contributions to variance of lifetime production of numbers of progeny following in utero exposures to specific x-ray dosages received at different stages in embryological development.

indicated that all three main effects, dose, strain, and sex were significant. In addition the Dose × Sex interaction was significant for both measures, and the Strain × Sex interaction was significant for number of litters. Concerning responses of specific genotypes, it was noted that after 320 r all of the inbred progeny were sterile, but only 50% of the hybrid progeny were sterile.

In addition to the analyses above, the data may be arranged to quantitate the effects of treatment at the different embryological ages when the levels of irradiation are fixed at 20 r, 80 r, and 160 r. Results of such analyses are given in Tables IV and V. Within the doses of 20 r and 80 r only the strain and sex effects are significant. Within 160 r, in addition to the significant strain and sex effects, there was a significant effect due to embryological stage and a significant Strain × Age interaction.

Estimation of components of variation

The amount of variation in lifetime reproductivities, as measured by number of litters and number of progeny can be partitioned into the amounts due to the

TABLE IV

Adult reproductive performance as influenced by the embryological age when the mice were irradiated; number of litters

Source of variation	Dose of irradiation in roentgens					
	20 r		80 r		160 r	
	df	M.S.	df	M.S.	df	M.S.
Embryological age (U)	3	11	3	22	2	85*
Strain (G)	8	94**	8	47*	8	145**
U × G	24	12	24	27	16	35*
Sex (F)	1	94**	1	779**	1	416**
U × F	3	14	3	11	2	52
G × F	8	23	8	40	8	30
G × U × F	24	30	24	26	16	23
Unaccounted for (E)	72	20	72	23	54	18

* 5% level of significance.
** 1% level of significance.

TABLE V

Adult reproductive performance as influenced by the embryological age when the mice were irradiated; number of progeny

Source of variation	Dose of irradiation in roentgens					
	20 r		80 r		160 r	
	df	M.S.	df	M.S.	df	M.S.
Embryological age (U)	3	106	3	1,625	2	4,878
Strain (G)	8	8,620**	8	4,298**	8	5,943**
U × G	24	675	24	1,538	16	2,691
Sex (F)	1	16,385**	1	24,258**	1	16,109**
U × F	3	248	3	358	2	3,803
G × F	8	1,410	8	1,100	8	834
G × U × F	24	1,149	24	1,518	16	1,847
Unaccounted for (E)	72	2,051	72	1,185	54	1,852

* 5% level of significance.
** 1% level of significance.

various effects and their interactions by utilizing estimated components of variance derived from an analysis of variance. It is assumed that the variations are due to components acting additively. The general mathematical model upon which the component analysis is based is:

$$Y_{ijkl} = u + g_i + t_j + (gt)_{ij} + f_k + (fg)_{ik} + (ft)_{jk} + (fgt)_{ijk} + e_{ijkl}$$

where u = the overall mean; $i = 1, 2, \ldots, 9$, the inheritance types; $j = 1, 2, 3$, (or 4 or 5), levels of irradiation; and $k = 1, 2$, the sexes exposed to irradiation. The general breakdown for the component analysis is given in Table VI. The components can be interpreted as follows:

G is the variation due to genetic or hereditary differences, T is the variation due to differences in effects of the dosage levels, and F is the variation between sexes. The interaction terms are interpreted as arising from the differential responses of the geno-types or sexes from one level of irradiation to the next. The term E, is considered due to uncontrollable variation, and represents random variation of individual differences of mice of the same sex within a litter given the same treatment. A separate component analysis was obtained for each of the embryological ages. Results are shown in Figures 1 and 2 for number of litters and number of progeny, respectively, where graphs showing the contributions of the different components of variance are plotted on the embryological ages when the irradiations were administered. Except following irradiation at 17½ days, the interactions involving sex: FG, FT and FGT, are small and have been combined within each of the embryological ages.

TABLE VI

Breakdown for the statistical analysis

Source of variation	d.f.	Components of variation
Among genotypes	8	$E + 2_jG$
Among dosages	$(j\text{-}1)$	$E + 18T$
Genotype × dosage	$8(j\text{-}1)$	$E + 2GT$
Between sexes	1	$E + 9_jF$
Sex × genotype	8	$E + {}_jFG$
Sex × dosage	$(j\text{-}1)$	$E + 9FT$
Sex × genotype × dosage	$8(j\text{-}1)$	$E + FGT$
Unaccounted for variation		E

* $j = 3$, 4, or 5, depending on which embryological age is analyzed.

The heredity influences on variance averaged slightly over 20% of the total variance for number of progeny and slightly under 20% for number of litters. The largest genotypic effects were observed following treatment on 14½ or 10½ days.

The relative importance of the dosage of irradiation components showed a slight increase from day 6½ through day 14½ increasing with increasing embryological age and reaching a maximum on day 17½ when approximately 30% of the total variance was accounted for by treatment effect. It should be stressed, however, that the age of 17½ days was the only stage in which mice survived a dose of 320 r for reproductive testing.

The interaction components between the levels of irradiation and genotypes on the different embryological ages were slight and never accounted for more than 10% of the total variation. The largest contribution to the variation of this interaction occurred on day 17½. The highly specific effects between the genotype and the ability of the treated fetuses to carry out adult reproductive activities was noted early in the observation that none of the inbreds that had been exposed to 320 r had any litters at all.

The sex components showed variations at all embryological ages averaging 12% of the total variation in number of litters over all embryological ages and 27% of the variation in number of progeny. The basic sex differences in reproduc-

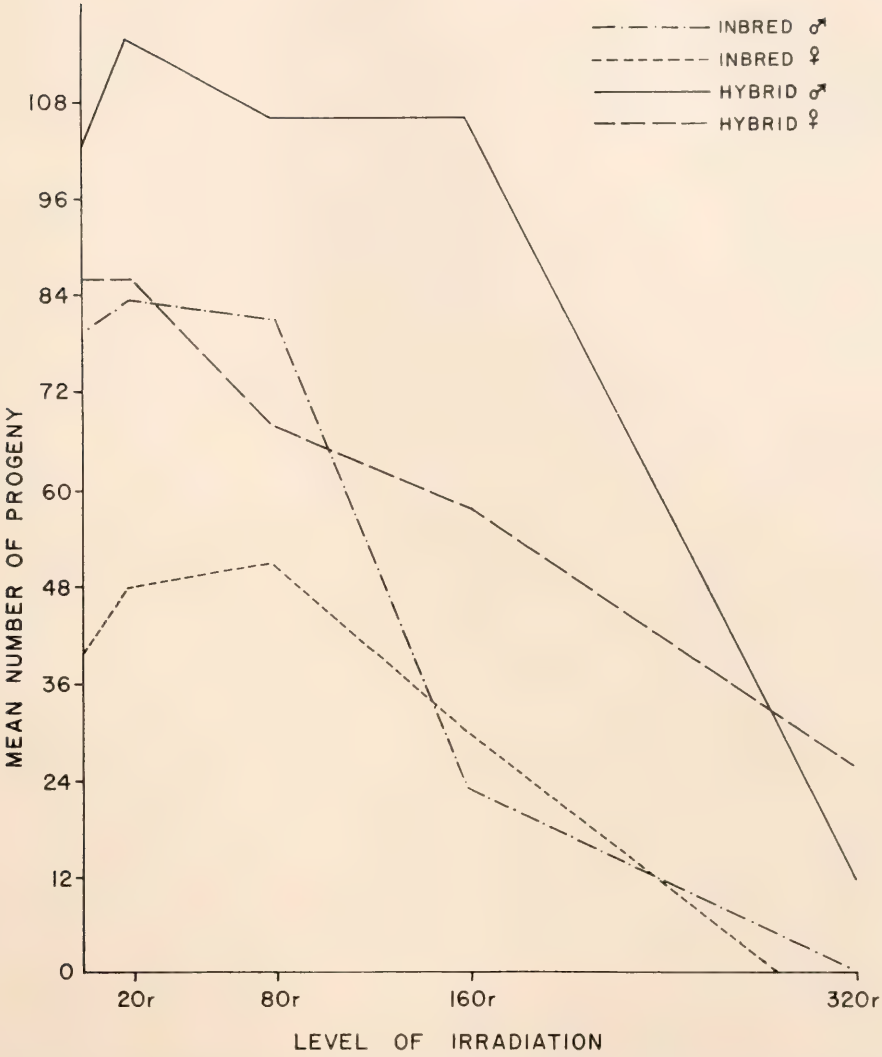

FIGURE 3. Mean number of progeny produced by inbred and hybrid mice following in utero exposures to specific x-ray dosages on day 17½ of gestation.

tive capacity are operational regardless of whether exposure to irradiation has occurred early or late in embryological development.

DISCUSSION

The results presented above demonstrate that embryological stage at the time of exposure to x-rays is most important in determining postnatal reproductive performance. Similar observations have been noted for other postnatal sequelae of embryonic irradiation. Although animals may appear normal at birth following embryonic irradiation, effects on morphological and physiological characteristics may become evident in later life. Effects of prenatal irradiation upon postnatal growth have been reported for several species, including the mouse (Russell, Badgett and Saylors, 1960; Nash and Gowen, 1962; Rugh, Duhamel, Chandler and Varma, 1964), the rat (Ershoff and Bavetta, 1958), and cattle (Parish, Murphree and Hupp, 1962).

Understanding of results of prenatal irradiation upon adult reproductive performance can be aided by a consideration of the events that are taking place at each of the embryological ages during normal development. Detailed descriptions of embryological development in the mouse may be found in other sources, including Rugh (1968). Day 6½ represents a stage shortly after implantation at about the time the mesodermal germ layer is beginning to appear. By day 10½ embryos are in a period of major organogenesis. Concerning the reproductive organs specificially, the primordial germ cells migrate from the yolk sac endoderm to the dorsal mesenteries and coelomic angles between days 8 and 12. The gonad primordia begin to form by day 10½, but the gonads remain undifferentiated until day 14 at which time testes and ovaries can be distinguished. Spermatogenesis becomes evident by day 14 and by day 14½ interstitial tissue and germinal epithelium are apparent. Development of the ovary is similar, and by day 14 oogonia in mitosis can be observed. Secondary sex characters begin to differentiate by day 17.

Several studies have been concerned with evaluating the response of the fetal reproductive system to irradiation. These studies have included both histological and functional aspects and have indicated that the reproductive system of the fetal female can tolerate more radiation than that of adult females. In contrast to the findings in adult animals, fetal males are more drastically affected by irradiation than fetal females. Rugh and Jackson (1958) observed the adult reproductive capacity of mice which had been exposed between days 15½ and 18½ of gestation to x-ray doses of from 50 r to 200 r. Peak sensitivity of the ovary occurred on day 16½ after 200 r. After day 16½ the fetal ovary appeared to be quite radioresistant. Russell, Badgett and Saylors (1960), irradiated mice on days ½ to 13½ and observed that irradiation on days 11½ and 13½ exerted the greatest effect on the reproductive capacity of the females.

In the present study, four different periods of pregnancy were sampled. The dose of 80 r is the highest dose that is represented at all stages. With this dose, day 10½ appears to be more radiosensitive than the other three ages. The mice that survived 160 r at 6½ days appeared fully fertile. Normality of surviving embryos from irradiation at early embryological stages has been noted for other characteristics as well. A dose of 160 r appears to have a slightly greater effect at 14½ days than at 17½ days, and the same relationship appears to hold true following a dose of 320 r.

Among fetal males, the study of Rugh and Jackson (1958) indicated that the peak of radiosensitivity was at 15½ days when an exposure of 200 r caused 45% of the males to be sterile, and fertile ones produced only 16% as many progeny as controls. With increasing embryological age fertility of males irradiated with 200 r improved towards control levels.

In the present study, at the lower doses, the response of the fetal testis appeared to be similar to that of the fetal ovary. No effect on the adult male reproductive system was noted among the survivors until a dose of 80 r at day 10½, which reduced fertility to 81% of that of control males, again indicating that the 10½ day stage is the most radiosensitive. There was a small but insignificant decrease in reproductive performance of males that had received 160 r at day 6½ but in other characteristics such as postnatal survival, growth, and lifespan, these animals were comparable to controls. The 14½-day stage appeared to be more sensitive than the 17½-day stage as evidenced by reproductive performance following a dose of 160 r. At the former stage this dose reduced fecundity to 85% of that of controls, whereas at the latter stage, this dose did not affect fecundity, but did appear to affect fertility. Compared to the fetal ovary the fetal testis at 14½ days was less sensitive. Following irradiation at day 17½ the females appeared relatively more affected than the males at lower doses, but following the highest dose used, were less affected.

The present studies confirm the well-established fact that genetic differences influence reproductive capacity. Although significant genotypic differences were found for both number of litters and number of progeny, the interaction involving genotypes and levels of irradiation were not significant in all cases indicating that the different genotypes were responding in a similar way to the effects of embryonic irradiation upon postnatal reproductive performance. Differential genetic responses to irradiation have been noted for these mice for growth (Nash and Gowen, 1962) and lifespan (Nash and Gowen, 1965). A possible difference in response between inbred and hybrid mice was noted following a dose of 320 r on day 17½. Although all of the inbred progeny were sterile, 50% of the hybrid progeny were fertile. In Figure 3 the response of inbred and hybrid mice following irradiation at day 17½ is given. Results indicate that hybrids are less affected by doses of 160 r and 320 r, fecundity among hybrids remaining relatively normal until a dose of 320 r is reached. Heterosis in radiation response of the adult reproductive system has been noted previously by Haverland and Gowen (1960) and Ehling (1964). Differences in response of inbred and hybrid embryos may be a reflection of slight differences in embryological stage of development at the time of irradiation as well as a reflection of inherent differences in susceptibilities to radiation-induced effects on the reproductive system.

Summary

1. Lifetime reproductive performance was observed in mice that had been irradiated at different stages of embryological development. Three genetically differentiated inbred strains of mice, BALB, K, and S, and all their possible hybrids, including reciprocals, were used. Pregnant females were exposed to single whole-body 250 pkv x-ray dosages from 20 r to 320 r on 6½, 10½, 14½ and 17½ days gestation, as timed from the appearance of a vaginal plug. Efforts were made to obtain two mice of each sex and inheritance type for each of the irradiation dose-embryological age treatment combinations. No progeny or not sufficient progeny were obtained following irradiation with 160 r or 320 r at embryological ages of 6½, 10½, and 14½ days of age. At 75 days of age treated mice were outcrossed to mice of the Z strain, and matings were maintained for the lifetime of the treated mice.

2. Adult reproductive performance was found to be related both to the age of the embryo at the time of irradiation and the level of irradiation. The embryological ages in order of increasing sensitivity were 6½, 17½, 14½, and 10½ days. The incidence of sterile matings was pronounced only following 160 r or 320 r at 17½ days. Significant effects on reproduction due to genotype and sex were observed at all ages. Survivors of irradiation at 6½ days had normal fertility and fecundity. A dose of 80 r at 10½ days reduced reproductivity to 76% of control values. Significant effects due to levels of irradiation were observed at both 14½ and 17½ days. Mice irradiated with 160 r at 14½ days produced only 72% as many progeny as controls. Following irradiation with 160 r at 17½ days, irradiated mice produced 83% as many progeny as controls. With a dose of 320 r at 17½ days the incidence of sterility was 67% and progeny production fell to 21%

3. Genetic differences in the response of the fetal reproductive system were most evident following irradiation on day 17½. Inbred progeny appeared to be more radiosensitive than hybrid progeny. The incidence of sterility after 320 r was 100% in inbreds compared to 50% for hybrids. Fecundity following 160 r was reduced in inbreds, but remained near normal in hybrids.

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WINTER POPULATION OF *LOLIGO PEALEI* IN THE MID-ATLANTIC BIGHT[1]

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The biology of the common Atlantic Coast squid, *Loligo pealei* (Lesueur, 1821), has been described principally from its summer, inshore populations (Verrill, 1882; Williams, 1909; Drew, 1911; Arnold, 1962; Haefner, 1964; Summers, 1968; and others). Migration of these populations is suggested by their annual appearance and disappearance from inshore areas. The offshore populations of *L. pealei* have been only partially described (Summers, 1967). This paper reports attempts to more precisely locate the winter distribution of *L. pealei* and estimate the abundance of this species.

Myopsid squid of the family Loliginidae are reported from the epipelagic zone in the sea, bounded by depths no greater than 200–400 m (Voss, 1967). They are not commonly found over deeper water and appear to be restricted to coastal margins and continental shelves around the world. *L. pealei* has been reported from New Brunswick, Canada (Stevenson, 1934) to Columbia (LaRoe, 1967), however, it is most often encountered between Cape Cod and Cape Hatteras on the East Coast of the United States (Verrill, 1882; LaRoe, 1967). Verrill (1882) reported this species in depths to 92 m and its eggs to half that depth. LaRoe (1967) gave a mean depth of 62 m for his collection of specimens, and Summers (1967) reported the peak offshore abundance of *L. pealei* at approximately 170 m.

The earliest inshore appearance of *L. pealei* in the vicinity of Woods Hole, Massachusetts is reported as: May 7, Bumpus (1898), and ranged from April 16 to May 7 for the years 1900–1910, (V. N. Edwards, cited by Sumner, Osburn and Cole, 1913). The offshore migration of this animal is less distinct and reported to take place in early November (Verrill, 1882). The latest trawl catches of *L. pealei* resulting from biweekly sampling near Woods Hole occurred on November 16, 1967 (Summers, 1968) and November 15, 1968 (Summers, unpublished).

Bigelow (1924, p. 113) suggested that *L. pealei* did not disappear from the inshore areas, and presumed that ". . . it passes the cold season inshore on the bottom." He was not aware of this species being reported from the offshore banks in summer nor from deep water. Though his writing indicates that he was experienced with this animal, Bigelow stated that he had not taken a single specimen of *L. pealei* in his "tow nets."

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The European species, *Loligo vulgaris*, is tentatively reported to have an annual coastwise migration in the North Sea (Tinbergen and Verwey, 1945); these authors state that descriptions of the autumnal migration and winter location of this squid are uncertain (p. 282). In the western Mediterranean Sea, *L. vulgaris* is reported to exhibit a seasonal vertical migration which brings it onshore in the spring and takes it offshore in the autumn (Mangold-Wirz, 1963, p. 140). With apparent reference to Verrill's work, Mangold-Wirz compared the latter migration with that of *L. pealei*. These two reports on *L. vulgaris* are based upon animals obtained from commercial fishing vessels and may incorporate some bias from these sources due to gear selectivity and the incidental nature of squid catches.

METHODS

Approximately five metric tons of *L. pealei* were collected with commercial North Atlantic otter trawl nets during four cruises listed in Table I. The first three cruises were spaced through the winter of 1966–1967 and the fourth cruise was made in the late winter of 1968, one year after the third cruise. Two cruises made by the R/V DELAWARE were parts of a single exploratory survey of the continental shelf and slope and do not represent duplication in sampling. Sampling conditions and cruise ranges are given in Table I. Cephalopod species were separated during the last three cruises and 97% of the individuals were *L. pealei*; the remainder were mostly *Illex illecebrosus*. Because of the dominance of *L. pealei* in these collections, I assume that data for "squid" catches on DELAWARE cruise 66–11 were representative for the one species.

TABLE I

Winter otter trawl surveys in the mid-Atlantic Bight

Year	1966	1967	1967	1968
Vessel	DELAWARE	DELAWARE	A. T. CAMERON	ALBATROSS IV
Cruise No.	66-11	67–1	130	68–3
Dates	XI 29–XII 15	II 2–II 21	III 16-IV 13	III 4–III 22
Area, Cape Hatteras to:	Hudson Canyon	Hudson Canyon	Georges Bank	Georges Bank
Otter trawl	#41	#41	#41/5[*]	#36
Mesh size, mm[†]	127–114	127–114	127–13	127–13
Headrope, m	24.4	24.4	24.4	18.3
Footrope, m	30.5	30.5	30.5	24.4
Tow speed, km/hr	6.5	6.5	6.5	6.5
Tow time, hr	1	1	$\frac{1}{2}$	$\frac{1}{2}$
Day/night	Day	Day	Day	Day & night
Depths, m	18–183	18–732	82–1465	27–366
No. of tows	42	54	35[‡]	159[‡]
No. *L. pealei* measured	None	1480	3352	3343
Per cent sexed	—	51	51	15

[*] A #41 otter trawl with an extension piece between the belly and the cod end.

[†] Stretched measure including one knot.

[‡] Number of tows within the sampling area shown in Figure 1. Additional stations were made to the north, and the A. T. CAMERON fished in waters deeper than 366 m.

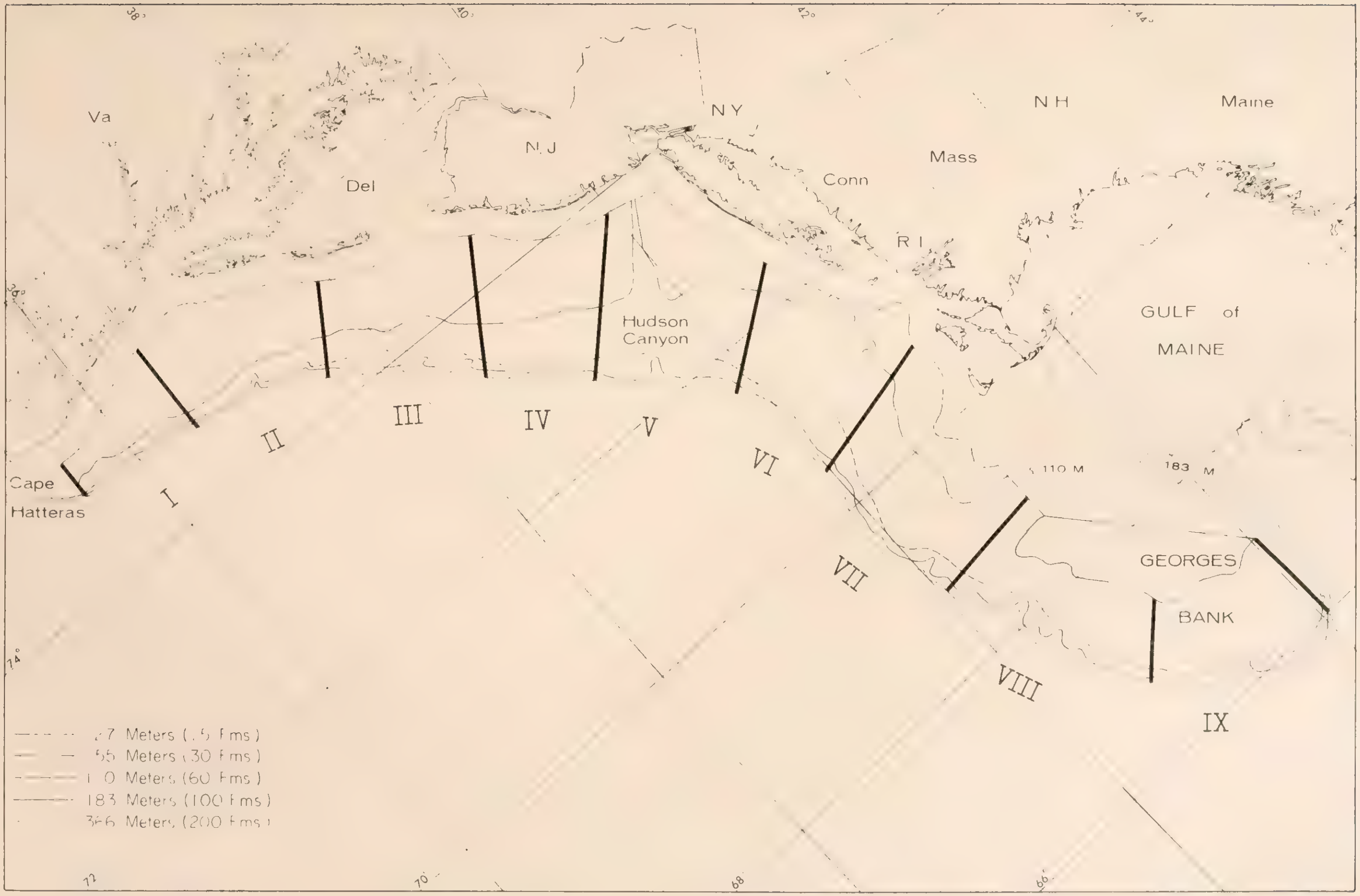
Delaware cruises 66–11 and 67–1 were conducted by the U. S. Bureau of Commercial Fisheries, Exploratory Fishing and Gear Research Base, Gloucester, Massachusetts. Catch data for these cruises were provided by Mr. E. D. McRae. Mr. Clarence Butt of the Fisheries Research Board of Canada measured and sexed samples of *L. pealei* during the second cruise. The Delaware otter trawl net was unlined and probably was not efficient in retaining small squid (see Summers, 1968). Sampling on these cruises was performed on transect lines perpendicular to the coast during daylight hours.

R/V A. T. Cameron cruise number 130 was conducted by the Fisheries Research Board of Canada, Biological Station, St. John's, Newfoundland. This cruise was primarily concerned with a study of the oceanic squid, *I. illecebrosus,* between Nova Scotia and Cape Hatteras (Mercer, 1969). The Scientist-in-Charge, Mr. M. C. Mercer, invited me to participate during part of the cruise between Cape Cod and Cape Hatteras. The Cameron otter trawl net was lined with a fine mesh liner and, as in the previous survey, sampling was restricted to daylight hours and to transect lines perpendicular to the coast.

R/V Albatross IV cruise 68–3 was conducted by the U. S. Bureau of Commerical Fisheries, Biological Station, Woods Hole, Massachusetts as a part of its regular groundfish survey program. At the invitation of Dr. M. D. Grosslein, I participated during the entire cruise. Later cruises extended the groundfish survey to the waters surrounding southern Nova Scotia. The Albatross IV used a lined otter trawl net which was smaller than those employed during the earlier cruises. Sampling conformed to a stratified random sampling design and operations continued both day and night (Grosslein, 1969). Geographical subdivisions from this design were used for the comparison of all of the cruise data resulting from the four cruises.

The region sampled (Figure 1) was confined to the offing between Cape Cod and Cape Hatteras (the mid-Atlantic Bight) with an eastward extension to include Georges Bank. The portion of the Bureau of Commercial Fisheries survey area used in this study is shown in the figure; this is subdivided by nine coastal zones (designated here by Roman numerals), each of which includes a set of strata corresponding to four depth zones. Depth zones are unequal in area and bounded by smoothed contours for depths of 27, 55, 110, 183 and 366 m. Thus, the region sampled is partitioned by a nine by four matrix except that the shallowest depth zone is not divided between coastal zones VIII and IX, an inconsistency which does not affect the present study. The continental shelf break occurs roughly at 120 m depth on Georges Bank and gradually decreases in depth to about 80 m off of Cape Hatteras; this separates approximately equal numbers of matrix cells either side of the shelf break.

Hydrographic measurements and catch records on the sampling cruises followed established practices for the various agencies and vessels. The catches of *L. pealei* were recorded by station (one tow per station) with some combination of data including weight, volume or number of individuals. On the last three cruises, length-frequency, sex and maturity were recorded for samples of up to several hundred individuals. Dorsal mantle lengths were measured from fresh squid to the nearest whole centimeter and maturity was established relative to arbitrary stages of gonadal development (Haefner, 1964; Summers, 1968).


Va
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Hudson Canyon
110 M
183 M
GEORGES BANK
Cape Hatteras
I
II
III
IV
V
VI
VII
VIII
IX
38°
40°
42°
44°
36°
74°
72
70
68
66
27 Meters (15 Fms)
55 Meters (30 Fms)
110 Meters (60 Fms)
183 Meters (100 Fms)
366 Meters (200 Fms)

FIGURE 1. The mid-Atlantic Bight and Georges Bank showing the sampling region and its subdivision by coastal zones (designated here by Roman numerals) and four depth zones.

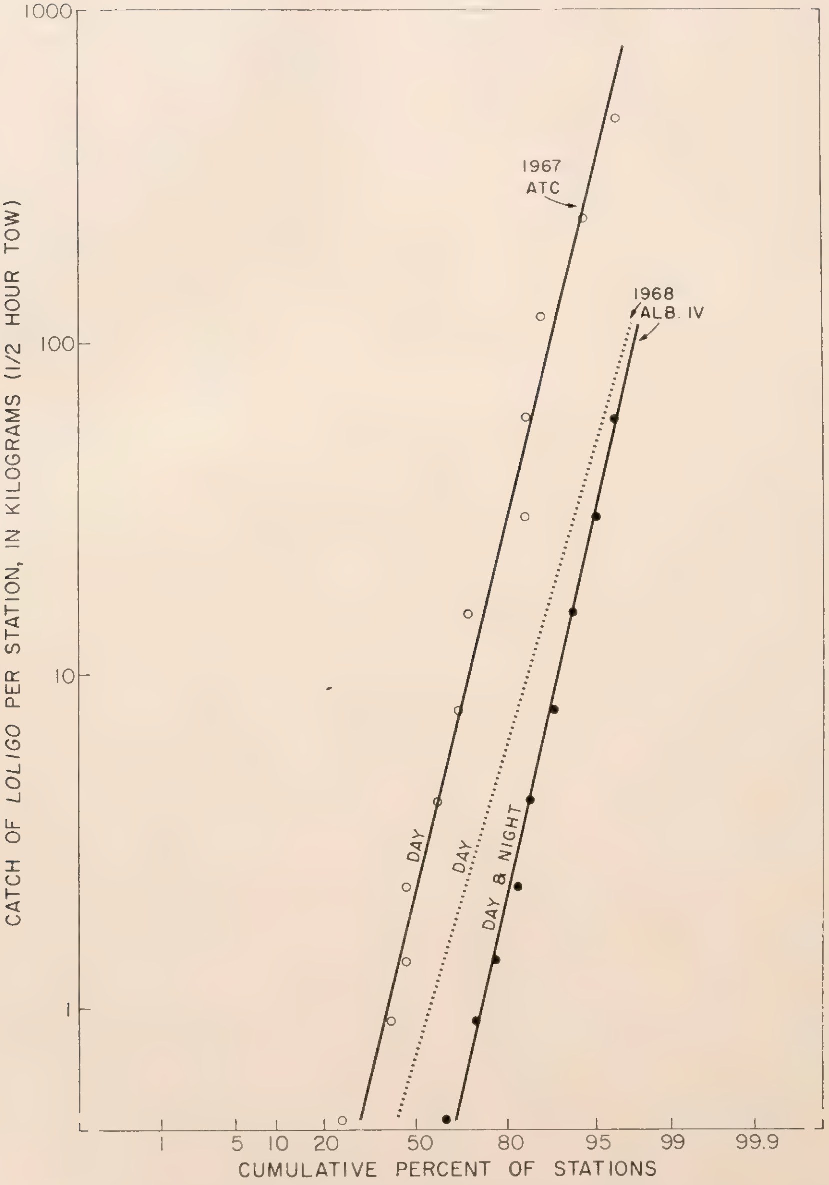

FIGURE 2. Log probability presentation of catches of *L. pealei* per tow for two late winter cruises. Straight lines in this figure represent lognormal frequency distributions of catches per tow. The 1967 cruise and dotted line data for the 1968 cruise are for daytime trawling only; the solid line for the 1968 cruise is approximately equally weighted for day and night trawling.

RESULTS

Because of my personal acquaintance with the cruises of the CAMERON and ALBATROSS IV, and since these are representative of the largest area and a single time of year, I will base most of the following results upon them and draw upon the DELAWARE data only for comparison.

Catches of *L. pealei* varied considerably from station to station for standardized tows. Frequency distributions of catches per tow showed a large positive skewness for all of the cruises. This, in itself, is a good indication of "patchiness" in the

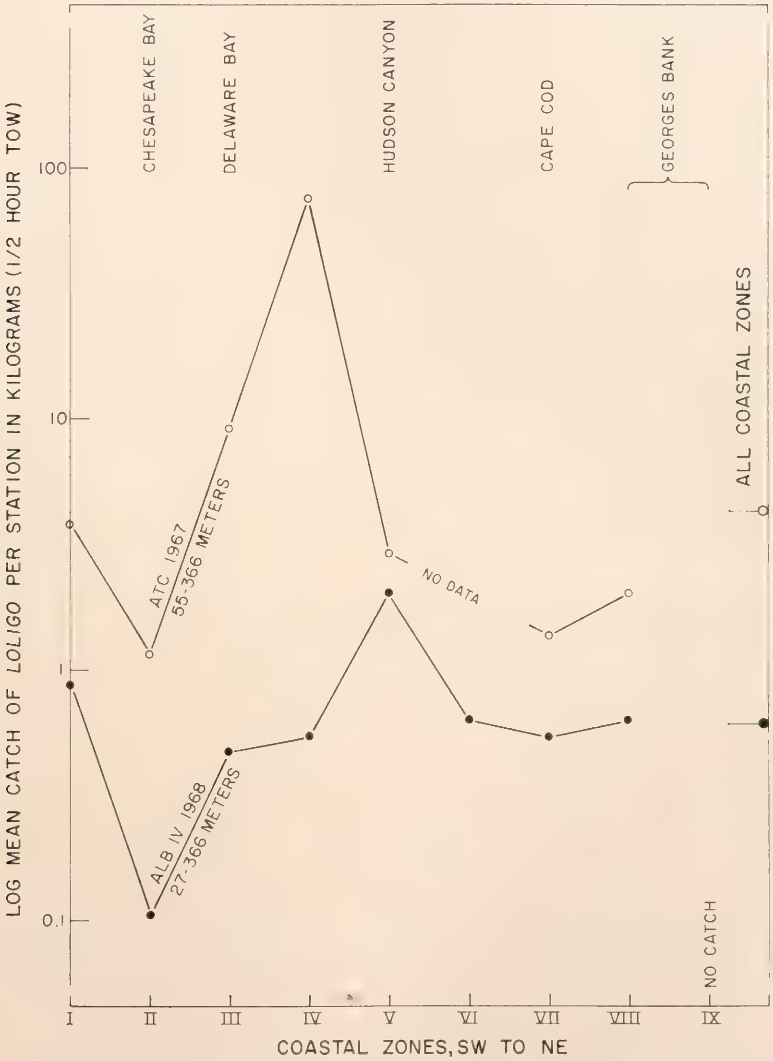

FIGURE 3. Log mean catches of *L. pealei* per tow for the nine coastal zones in 1967 and 1968. Overall log mean catches are shown at the right margin.

distribution of *L. pealei,* since a uniform distribution should produce a symmetrical catch per tow histogram. Figure 2 shows a log probability presentation of the catch per tow data for the last two cruises; straight lines on this figure represent lognormal catch per tow histograms. The straight line fit of data points in Figure 2 suggests that the catch data can be treated by normal statistics after a log transformation. (Lognormal models are useful in biology where logs of the variate are normally distributed. In this application, the biomass of *L. pealei* resulting from a standardized tow forms an approximate geometric series which can be fitted by a lognormal model.)

As is apparent from Table I, the two cruises represented in Figure 2 had substantial differences in sampling, including years, gear, depths and hours of sampling. Since both cruises extended through the time of the vernal equinox, it was practical to separate day catches from night catches at arbitrary mid-tow times of 6:00 AM and 6:00 PM. The solid line for 1967 and the dotted line for 1968 represent day catches as shown in Figure 2.

The remaining differences in sampling and the subsequent analysis of factors relating to the distribution and abundance of *L. pealei,* requires some average measure of the catch per tow data. The lognormal catch per tow histogram suggests a log mean for this purpose, or the mean of the log transformation of the

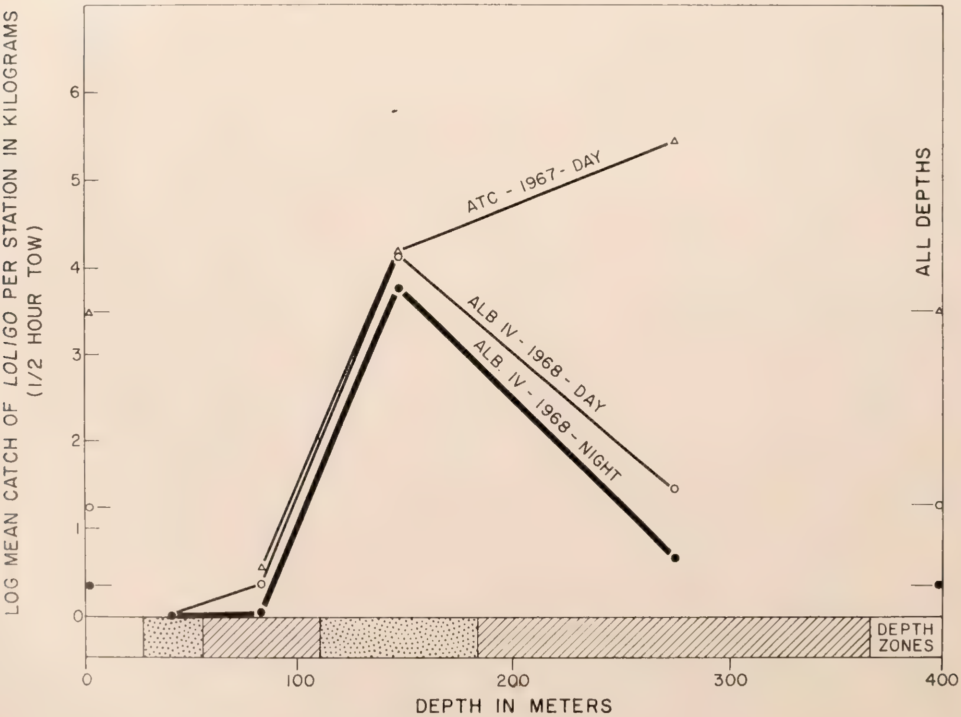

FIGURE 4. Log mean catches of *L. pealei* per tow for the four depth zones in 1967 and 1968. The 1968 data is separated into day and night portions and overall log mean catches are shown at the right margin.

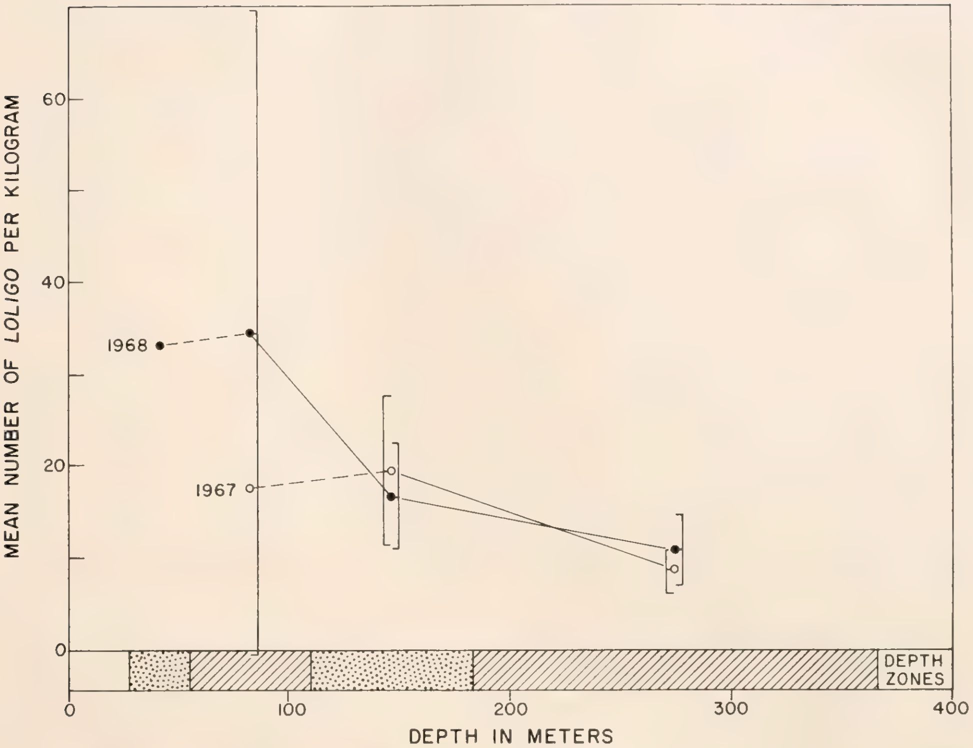

FIGURE 5. Mean number of *L. pealei* per kilogram for samples from the four depth zones in 1967 and 1968. Samples were a minimum of one kilogram in size. Bars represent one standard deviation either side of the means. Dashed lines extend to means of a single sample.

data. An arithmetic mean of the untransformed data would be greatly affected by the skewness of the data distribution and more erratic than a log mean.

Figure 3 shows the log mean catches of *L. pealei* in 1967 and 1968 for each of the nine coastal zones. Moving from southwest to northeast, there were three features in common for these two years: 1. There was a minimum abundance of squid in coastal zone II off of Chesapeake Bay. 2. The maximum abundance occurred in the center of the mid-Atlantic Bight (zone IV 1967; zone V 1968). 3. There were no catches of *L. pealei* in zone IX, on the eastern end of George Bank. The last observation was supported by nine extra tows made on the eastern end of Georges Bank during the 1968 cruise. Figure 3 indicates a high probability that a single population of *L. pealei* exists between southern Georges Bank and at least Cape Hatteras in the late winter.

Log mean catches of *L. pealei* in 1967 and 1968 are shown by depth zones in Figure 4. The 1968 data are separated into day and night portions in this figure. Very few squid were taken shallower than 110 m, or over the continental shelf. Peak abundance for the 1968 cruise (and for the two DELAWARE cruises) occurred in the 110–183 m depth zone; the 1967 cruise had an even greater catch in the deepest zone. Neither DELAWARE 67–1 nor CAMERON succeeded in catching

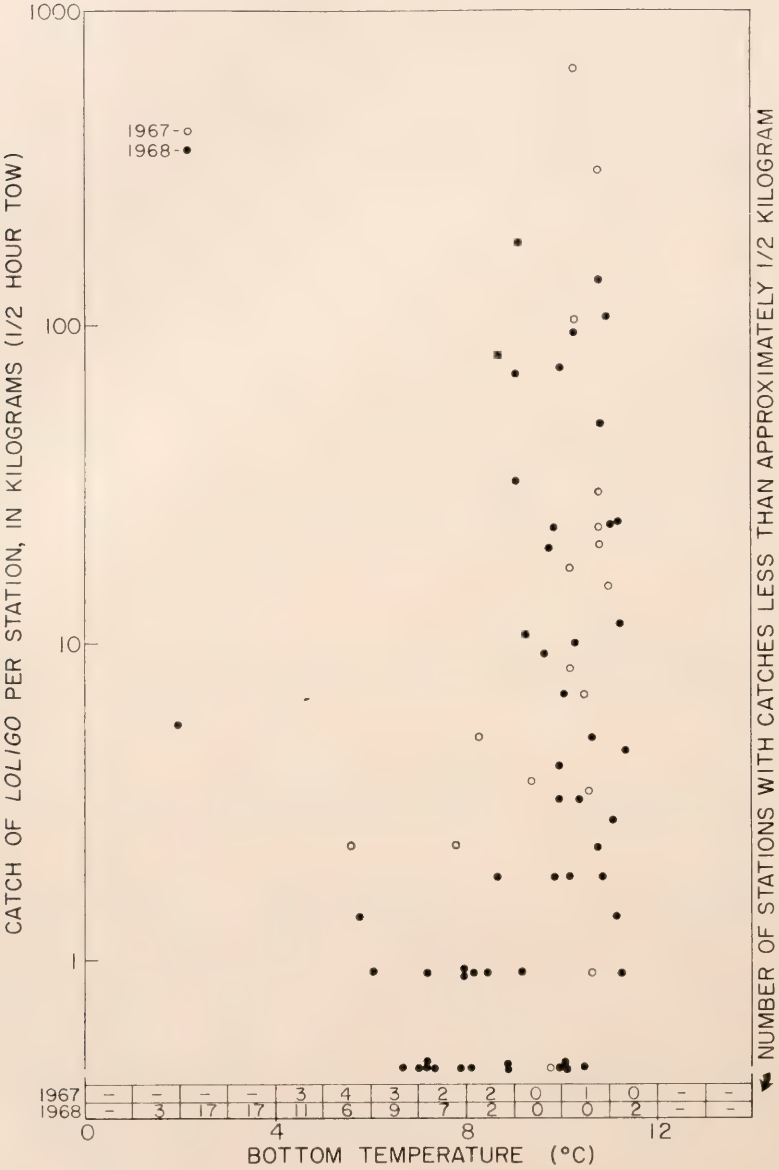

FIGURE 6. Catches of *L. pealei* per tow in 1967 and 1968 as a function of bottom temperature. Numbers of tows resulting in catches less than one-half kilogram are tabulated by year at the bottom of the figure.

L. pealei below a minimum tow depth of 366 m. Day catches were uniformly higher than night catches in 1968 and the former were remarkably similar to the 1967 data in the middle depth zones.

As previously reported (Summers, 1967) individual specimens of *L. pealei* were larger at greater depths. Figure 5 shows this relationship for measured samples of one kilogram or larger in 1967 and 1968. The trend of these data indicates a decreasing number of squid per kilogram with depth. The standard deviation shown for the 55–110 m depth zone (1968) is highly exaggerated because different size classes were found in that zone. The remaining standard deviations are approximately 30–40% of the means. The data may be converted to dorsal mantle lengths by a rough rule of thumb: Five centimeter *L. pealei* are approximately 44/kg, 10 cm *L. pealei* approximately 22/kg, 15 cm approximately 11/kg, 20 cm approximately 5.5/kg, and so on. This rule applies to fresh animals of mixed sexes, but not to squid less about 5 cm dorsal mantle length nor mature animals all of one sex.

Measured specimens of *L. pealei* ranged in dorsal mantle length 4 to 30 cm, 3 to 34 cm and 2 to 29 cm for the cruises DELAWARE 67–1, CAMERON and ALBATROSS IV, respectively. Median dorsal mantle lengths varied according to the proportions of different size classes in the measured samples; they were 12, 17 and 11 cm, respectively, for these cruises. Almost all of the female squid taken in March and April were sexually immature. Small males were mostly immature and larger ones were mostly mature (had spermatophores), the break coming at 17 cm dorsal mantle length at which size 50% of the males were considered mature. Using the same indices, Haefner (1964) found 50% of both sexes mature at a size of 8 cm in the summer time. These data suggest an annual regression of sexual development in *L. pealei* similar to that described for *L. vulgaris* (Tinbergen and Verwey, 1945).

Sex ratios in the catches were generally close to one to one, though a chi-square test applied to the larger CAMERON catches gave a probability of less than 10% that the sexes were in equal numbers. The weighting in this test was borne by a few disparate tows and could indicate local sorting of the sexes. Due to dimorphism, the sexes were not equally common for all size intervals. Attempts to separate the size classes for each sex were not conclusive. It was apparent, however that the growth of current year class *L. pealei* continues at its previously reported rate with little diminution to March and April of the first winter (see Summers, 1968). These squid had grown to a size of 12 to 14 cm dorsal mantle length in nine months. A second group of current year class *L. pealei* was found in shallow depths, south of Hudson Canyon; these had a mean size of about 8 cm dorsal mantle length.

Log mean catches of *L. pealei* are shown as a function of bottom temperature in Figure 6. It is apparent from this figure that there is a pronounced reduction in the catches at bottom temperatures below 8° C. Data from the DELAWARE cruises are not shown in the figure because these cruises were made earlier in the season when bottom temperatures were generally above 8° C.

Figure 7 shows the range of bottom temperatures for the nine coastal zones in 1967 and 1968. It should be noted that bottom temperatures increased with depth in the late winter and that 8° C temperatures were encountered everywhere in this range except in coastal zone IX. By comparison with Figures 3 and 4, it can be seen that the major catches of *L. pealei* were restricted to bottom temperatures of 8° C or higher.

The foregoing presentation of results has contrasted log mean catches of

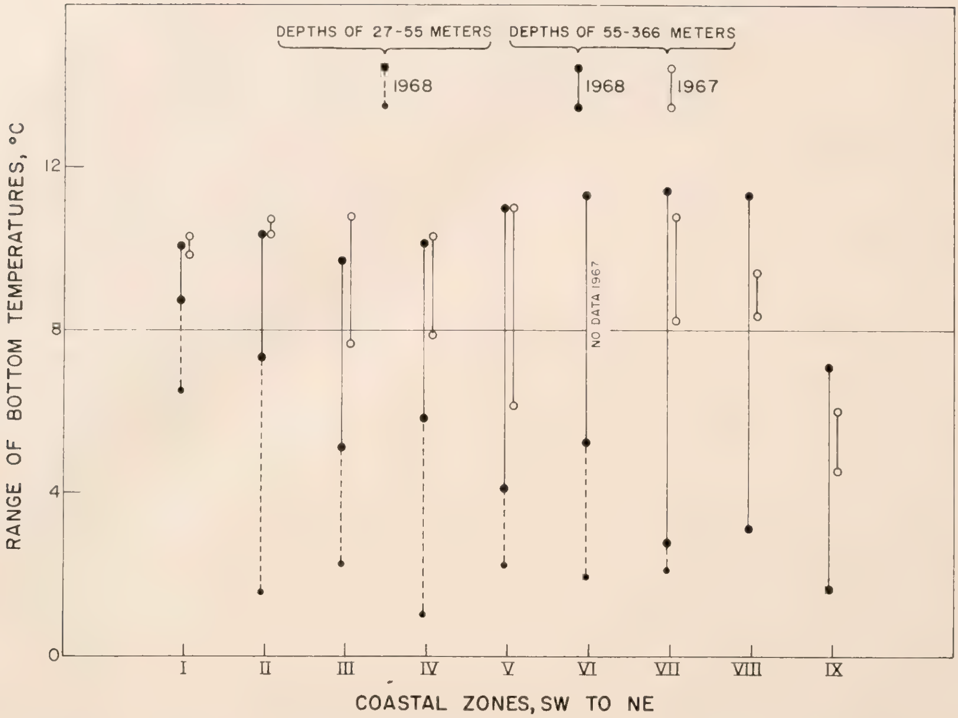

FIGURE 7. Ranges of bottom temperatures for the nine coastal zones as determined from bathythermograph records at the sampling stations during the late winters of 1967 and 1968. An 8° C isotherm is shown in the figure.

L. pealei without determining the significance of their differences. Furthermore, the CAMERON and ALBATROSS IV cruises were treated as measurements for two years, when in fact data for the two years is confounded with the two vessels and their respective gear. The year-gear factor cannot be separated with the data at hand, but the significance of this and other factors can be determined by a statistical test.

The variable which was chosen for analysis was the log transformed catch of *L. pealei* per tow for daytime trawling. The model was factorial with prime factors: depth, coastal position and the inseparable combination of year-gear. In order to make the analysis manageable, the coastal position factor was reduced to three levels by pooling coastal zones in groups of three (I-III, IV-VI and VII-IX). The depth factor consisted of three depth zones (55–110, 110–183 and 183–366 m). The year-gear factor was composed of two levels which might be designated: 1967-CAMERON and 1968-ALBATROSS IV. Through the use of a table of random numbers, data were selected to provide two replications per cell, thus discarding some of the available information at the expense of simplification. There were no data for the cell VII-IX, 55–110 m, 1967-CAMERON; these were supplied by using equivalent 1968-ALBATROSS IV data in their place. The substitution should not be troublesome because this was an especially well sampled area in 1968. Of 21 tows in that year, 12 were devoid of *L. pealei* and the remainder produced minor catches. It seems unlikely that the CAMERON would have caught large quantities of squid in this region in 1967.

The selected data were submitted to an analysis of variance with the following results: Coastal position and depth were found to be significant with better than 99% confidence. The remaining prime factor, year-gear, and all interactions were not demonstrably significant. The significance of coastal position is not readily apparent (Fig. 3) unless it is appreciated that pooling the coastal zones enhanced the contrast between the abundance of squid in the central mid-Atlantic Bight and the two ends of the Bight. The depth preference of *L. pealei* is apparent in Figure 4. The lack of significance in the year-gear factor does not imply equality between the two levels, but suggests that the years were not radically different. The actual surveys were more highly structured than the three coastal positions imply. This represents a restriction on the randomization of the error term in the analysis of variance, and probably made the results of the test conservative.

DISCUSSION

Estimates of relative abundance

It is possible to treat otter trawl catches as quantitative samples and estimate the relative abundance of a species from them. This allows a more direct comparison of biomass or numbers of individuals than contrasts of means.

Results of daytime trawling shown in Figure 2 indicate that the CAMERON (with its larger trawl) outfished the ALBATROSS IV and that the disparity was greater with larger catches. This corroborates results obtained by the use of different sized otter trawls inshore, assuming the squid population was substantially the same, and indicates further quantification of trawl catches.

The Bureau of Commercial Fisheries recently installed instruments on otter trawls which measure dimensions of the trawl while underway. In one application, Bruce (1968) measured the horizontal opening of #36 and #41 otter trawls to be 10 and 14 m, respectively. With the towing speed (6.5 km/hr) and towing time (1/2 hr), it is possible to calculate the bottom area swept by the trawl in one standard tow. Multiplying the log mean catch of *L. pealei* in any cell in the sampling matrix by the ratio of cell area to swept area results in an estimate of relative abundance of *L. pealei* (by weight) for that cell. This estimate is almost certainly lower than the population because of: squid escaping from the trawl, avoidance of the trawl, squid present above the vertical opening of the trawl and loss of specimens during the hauling of the trawl.

These losses, and probably others, make quantification of an otter trawl especially difficult. The end result is that the estimates of relative abundance probably grossly underestimate the natural population.

Overall estimates of relative abundance were calculated to be 3.4 and 2.1×10^6 kg of *L. pealei,* respectively, for the years 1967 and 1968. Owing to differences in bottom area of the various depth zones, the largest relative biomass was in the 110 to 183 m depth zone which accounted for 1.9 and 1.6×10^6 kg of *L. pealei*, respectively, for these same years. Numbers of indivduals were determined from the relative abundances through the use of results shown in Figure 5. Thus, the sampling gave overall estimates of 5.7 and 4.5 × 10^7 squid, respectively, for the years 1967 and 1968. As these estimates apply to the late winter, one month before the earliest inshore appearance of *L. pealei* near Woods Hole, and there is little likelihood of continuing recruitment, they should represent a minimal annual population. Additionally, the log mean data are conservative and produce lower estimates of relative abundance than arithmetic mean catches per tow.

Temperature

There is a striking correlation between the relative abundance of *L. pealei* and the occurrence of bottom temperatures in excess of 8° C. This mechanism alone could explain why *L. pealei* was not found in coastal zone IX and why it was taken in the 27–55 m depth zone only in coastal zones I and II (1968). Late winter bottom temperatures in the sampling region generally increased with depth to a maximum of about 10° C at 150 m depth. Similarly, temperatures over the continental shelf decreased from southwest to northeast along the mid-Atlantic Bight. These trends are shown in Figure 7.

Annual cycles of bottom temperature for the continental shelf between Nova Scotia and Hudson Canyon were reported by Schopf (1967). These data indicate that offshore bottom temperatures north of Georges Bank do not ordinarily rise above 8° C at any time of year. Thus, the appearance of *L. pealei* in the Bay of Fundy very likely is the result of seasonal inshore migrations (see Bigelow, 1924; Stevenson, 1934). Ten year mean water temperatures measured at the Portland (Maine) and Boston (Massachusetts) lightships indicate that inshore surface water temperatures exceed 8° C from late May to late November (D. F. Bumpus, personal communication). These vessels are situated over 46 and 30 m of water, respectively, and have measured bottom temperatures in excess of 8° C only during the last 2½ months of that period. Therefore, an 8° C temperature restriction of *L. pealei* would greatly limit the movements and breeding season of this animal north of Georges Bank.

Mr. Bumpus' data indicate that long term mean surface and bottom water temperatures in the vicinity of Woods Hole both exceed 8° C from early May to late November. This corresponds to the inshore season of *L. pealei* previously cited and confirms the importance of this temperature restriction. The period of temperatures in excess of 8° C is longer as one moves west and south from Woods Hole along the mid-Atlantic Bight; this suggests the possibility of a longer inshore season of *L. pealei* in that region. It could also account for the small squid taken south of Hudson Canyon in the late winter by suggesting that they result from an extended breeding season in the southern part of the mid-Atlantic Bight. *L. vulgaris* is reported to have a single breeding season in the North Sea (Tinbergen and Verwey, 1945) and a double breeding season in the western Mediterranean Sea (Mangold-Wirz, 1963); a similar extension in the breeding season of *L. pealei* could produce this effect in the mid-Atlantic Bight.

Biological implications

The migration of *L. pealei* cannot be fully described without tagging studies, but the present report on distribution and abundance can be compared with the literature on summer squid populations to indicate general limits of the migratory pattern. The northern range limit of *L. pealei* in winter is 600 km south of the reported summer range limit, and a longer migrational distance is suggested if the animal must remain inshore to avoid water temperatures below 8° C. No other evidence exists for a coastwise migration except that coastwise components in the migration would be beneficial in dispersing squid found in the central mid-Atlantic Bight in the late winter. The bulk of the *L. pealei* population is found just below the continental shelf break in late winter and these animals must migrate up to 200 km to come inshore in the spring. Hence, *L. pealei* must have a strong onshore component in its migration and probably migrates considerable distances twice each year.

It is not appropriate to place *L. pealei* in a demersal assemblage on the basis of trawl catches. Animals taken in trawls are selected by size according to species and do not necessarily represent single communities. Co-occurrence of species on the fishing grounds is the subject of continuing study by fisheries agencies and better examined with more data than is available for this study. The fact that *L. pealei* is taken with otter trawls indicates that it is demersal at least part of the time and the difference between day and night catches (Fig. 4) suggests that it disperses vertically at night.

The author wishes to acknowledge the many people who have assisted in the gathering and analysis of data presented in this report. From the Fisheries Research Board of Canada these include Mr. M. C. Mercer, Mr. Clarence Butt, the officers and crew of the A. T. CAMERON and the scientific party on cruise #130: Messrs. G. Dawe, E. M. LeGrow, E. E. Squires, W. R. Squires, P. R. Stokes, R. J. Tizzard and G. E. Tucker. From the U. S. Bureau of Commercial Fisheries, Mr. E. D. McRae, Drs. R. L. Wigley and M. D. Grosslein, the officers and crew of ALBATROSS IV and the scientific party on cruise 68–3, especially Messrs. F. E. Nichy, R. R. Marak and H. Stern, Jr. Finally, from the Systematics-Ecology Program, Miss J. M. Reinhart, who stood watches on the last cruise. Drs. N. R. Sinclair, M. R. Carriker and C. F. E. Roper read and criticized the manuscript.

SUMMARY

1. The distribution and relative abundance of *L. pealei* is reported from otter trawl sampling in the mid-Atlantic Bight during the late winter.

2. A lognormal frequency distribution of catches per tow suggests a "patchy" spatial distribution of *L. pealei*.

3. This species ranged from the southern edge of Georges Bank at least as far as Cape Hatteras in depths from 28 to 366 m.

4. Specimens of *L. pealei* were found to be especially concentrated in the central mid-Atlantic Bight at depths of 110-183 m.

5. The sizes of individual squid generally increased with depth, though there were mixtures of size classes in the 55–110 m depth zone.

6. *L. pealei* was found restricted mainly to water temperatures of 8° C or higher. This is related to the migration and summer distribution of the animal.

7. Conservative estimates of relative abundance of *L. pealei* in the mid-Atlantic Bight for the late winters of 1967 and 1968 are approximately 5×10^7 individuals.

8. Biological observations indicate an annual regression of sexual development during the winter months, the possibility of an extended breeding season south of Hudson Canyon and a continued rapid growth rate of current year class squid through the first winter.

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REAGGREGATION OF INSECT CELLS *IN VITRO*. I. ADHESIVE PROPERTIES OF DISSOCIATED FAT-BODY CELLS FROM DEVELOPING SATURNIID MOTHS

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Cells dissociated from the fat-body of developing saturniid moths undergo rapid reaggregation when cultured in insect blood (Walters and Williams, 1966). The phenomenon as a whole is reminiscent of the self-reconstruction of tissues from the dissociated cells of sponges and vertebrate embryos (Wilson, 1907; Townes and Holtfreter, 1955; Humphreys, 1963; Moscona, 1965). The important difference is that the cells of the dissociated fat-body lack any intrinsic motility or power of locomotion.

By means of time-lapse cinematography, it was possible to show that the dissociated fat-body cells are drawn together by certain motile hemocytes which are regularly present in the hemolymph of developing moths. These "plasmatocytes" were seen to crawl about and frequently adopt a single shape with their elongate processes adhering to two or more fat-body cells or groups of cells which were then pulled together by the active contraction of the plasmatocytes.

In stationary cultures of dissociated fat-body cells, reaggregation was completely blocked when the plasmatocytes were removed or otherwise inactivated. Cultures lacking all hemocytes could be made to reaggregate, however, by subjecting them to gentle agitation. The fat-body cells collided at random and cohered to form large aggregates. This finding strongly implies an intrinsic adhesiveness between fat-body cells. In the experiments reported here, the phenomenon has been further examined in order to identify the factors prerequisite to this mutual adhesion.

MATERIALS AND METHODS

Experimental animals

Coccoons of the Polyphemus, Cynthia, and Cecropia silkworms (*Antheraea polyphemus, Samia cynthia,* and *Hyalophora cecorpia;* family Saturniidae) were obtained from dealers and stored in the cold (Polyphemus and Cecropia at 5° C; Cynthia at 8° C). After at least three months of chilling, the pupae were removed from their cocoons and placed at room temperature (22°–25° C). The onset and subsequent stages of adult development were identified by externally visible characters according to the timetables prepared for Polyphemus (Nüesch, 1965; Walters, 1967), Cynthia (Williams, 1968), and Cecropia (Schneiderman and Williams, 1954).

Dissociated fat-body cells

Pupae were selected on the third to fifth day after the initiation of adult development. A scalpel incision was made in the mid-abdomen, and by gentle pressure large masses of fat-body were extruded through the wound. The tissue was excised and transferred to centrifuge tubes containing a small volume of physiological saline (Weevers, 1966). Dissociation into single cells was accomplished by drawing the tissue through a Pasteur pipette (bore 1 mm). The resulting suspension was divided among a number of tubes. The cells were collected by centrifugation at the low speed of a clinical centrifuge for one minute. This procedure effectively separated the heavy fat-body cells from hemocytes and debris. The fat-body cells were washed once with Weevers's solution and immediately resuspended as described below.

Assay for reaggregation

Hemolymph obtained from diapausing pupae through an incision in the facial region was collected in one or more centrifuge tubes containing crystals of streptomycin and phenylthiourea (Schmidt and Williams, 1953) and then centrifuged at 1500 *g* for 10 minutes to remove all hemocytes.

TABLE I

Effect of plasma from different sources on the reaggregation of fat-body cells

Source of plasma	Developmental stage of plasma donor	Source of fat-body cells	Reaggregation after 2 hours
Polyphemus	Diapausing pupa	Polyphemus	maximal
Polyphemus	Early development	Polyphemus	maximal
Polyphemus	Late development	Polyphemus	maximal
Polyphemus	Diapausing pupa	Cynthia	maximal
Polyphemus	Diapausing pupa	Cecropia	maximal
Cynthia	Diapausing pupa	Cynthia	maximal
Cynthia	Early development	Cynthia	maximal
Cynthia	Diapausing pupa	Polyphemus	maximal
Cecropia	Early development	Cecropia	maximal
Cecropia	Diapausing pupa	Polyphemus	maximal

In each series of assays, one aliquot of fat-body cells was resuspended in plasma diluted with an equal part of Weevers's solution; the remaining aliquots were resuspended in various experimental media described below. In each case, sufficient solution was added to give a cell density of *ca.* 100 per μl, as determined by hemocytometer count.

Reaggregation was assayed by the technique described by Moscona (1961). One to 1.5 ml of cell suspension was placed in a 10-ml Erlenmeyer flask and subjected to gentle swirling at 65 rpm on a rotary shaker (radius of displacement 1 cm; A. H. Thomas Co., Philadelphia). The suspensions were allowed to rotate for 2 to 3 hours, and the degree of agglutination was scored as described in Table II. Except where otherwise noted, the experiments were performed on the cells and plasma of Polyphemus.

Table II

Effect of plasma concentration: degree of reaggregation of fat-body cells in pupal plasma diluted with physiological saline

(Identical results were obtained for both Polyphemus and Cynthia)

Amount of plasma in medium	Degree of aggregation after 2–3 hours
≧40%	+++ Maximal; small number of large clumps (average diameter 1 mm), almost no single cells.
20%	++ Moderate; large to medium clumps, a few single cells.
10%	+ Partial; medium to small clumps, half of cells remain single.
5%	(+) Trace; many tiny clusters of only 2–6 cells each, most cells remain single.
2.5%	0 No apparent agglutination
0%	0 No apparent agglutination

Results

Changes in the fat-body during adult development

The fat-body of diapausing pupae consists of numerous ribbons of branching and anastomosing tissue which surround the viscera and fill most of the abdomen and thorax. On histological examination, individual cells are found to be about 50 μ in diameter; each is characterized by a central, round nucleus and a cytoplasm charged with large granules which stain metachromatically with toluidine blue. The cells fit intimately together, and the tissue as a whole is surrounded by a thick basement membrane.

Two or three days after the initiation of adult development, the basement membrane disappears. Simultaneously, the strands of tissue fuse into an amorphous mass from which individual cells are easily dissociated by any mechanical stress (*cf.* Ishizaki, 1965; Krishnakumaran, Berry, Oberlander and Schneiderman, 1967).

During the three weeks which the pupal-adult metamorphosis requires at 25° C, the fat-body becomes progressively reduced in volume and restricted to the abdomen. After the tenth day of development, the tissue takes the form of numerous small clusters of cells adhering to other organs and especially to the tracheae. Shortly before the emergence of the adult moth, the stranded morphology of the fat-body is restored; the tissue lies mainly around the abdominal tracheae and is once again invested by a prominent basement membrane. The individual cells are greatly shrunken; they show irregular nuclei and a cytoplasm virtually devoid of granules.

Only during the first third of adult development can the fat-body be readily dissociated. Attempts to dissociate pupal or adult fat body proved unsuccessful even after prolonged immersion in calcium- and magnesium-free saline solutions containing the chelating agent ethylenediaminetetraacetate (EDTA).

Reaggregation in suspensions of fat-body cells

When dispersed fat-body cells were suspended in plasma and subjected to continuous rotary agitation, they promptly began to cohere. As reaggregation com-

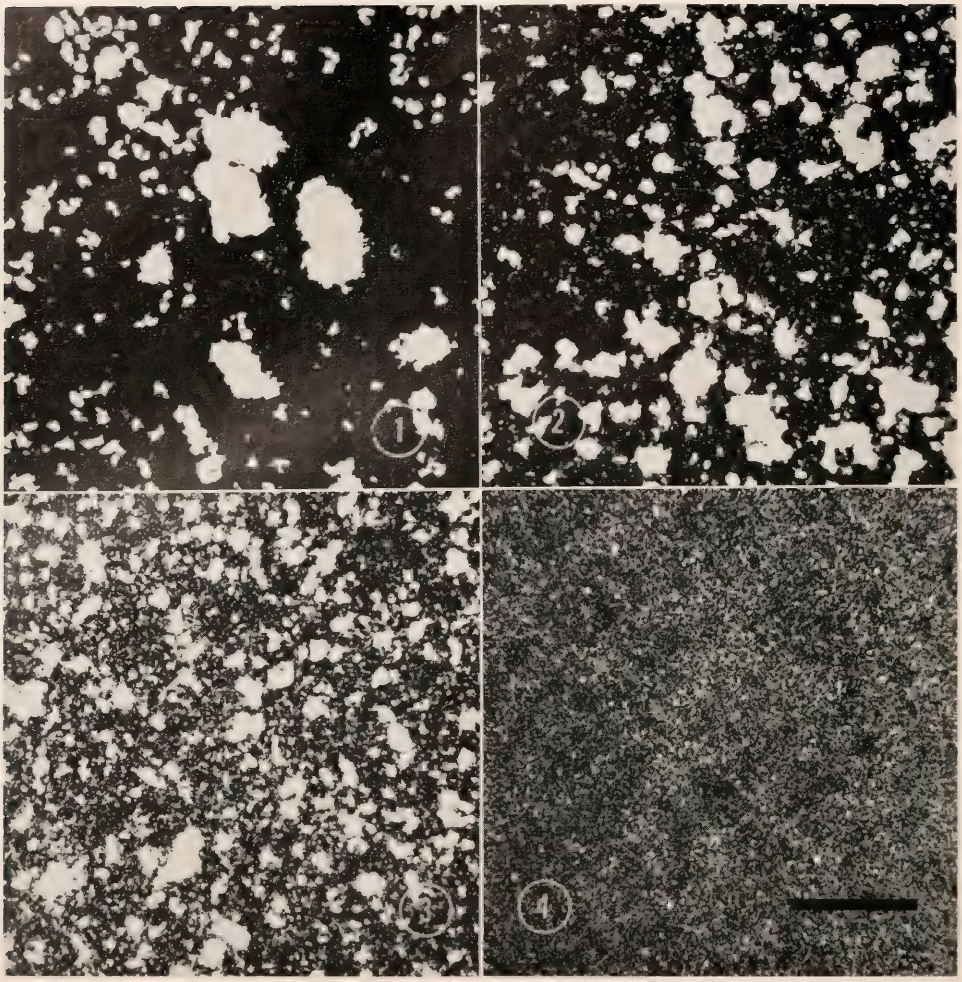

FIGURE 1. Aggregates of fat-body cells which formed after 2 hours of rotation in 40 per cent plasma. (Maximal aggregation.)

FIGURE 2. Same, in 20 per cent plasma. (Moderate aggregation.)

FIGURE 3. Same, in 10 per cent plasma. (Partial aggregation.)

FIGURE 4. Unaggregated cells in Weevers's solution. Black bar represents 4 mm; all Figures at the same magnification.

menced, the suspension became less turbid. Tiny clusters of cells formed and gradually coalesced until maximal aggregation was attained in the course of 2 or 3 hours at room temperature. Fully formed aggregates were compact, spherical, and ranged from a few tenths to more than two millimeters in diameter (Fig. 1).

The aggregates could be again dissociated into single cells by drawing them through a Pasteur pipette. When returned to the rotary shaker, the cells underwent reaggregation as before. Histological examination of fixed and sectioned aggregates revealed intimate cellular contact within each cluster.

Role of cellular metabolism

Dissociated fat-body cells were suspended in plasma and placed on a rotary shaker in the cold (2°–3° C). Reaggregation occurred almost as rapidly as at room temperature. In this case, however, the final aggregates were irregular in shape, and individual cells cohered loosely.

A similar result was observed when cell suspensions were treated with 10^{-3} M 2,4-dinitrophenol—a concentration which fully inhibited cellular locomotion and reaggregation in stationary cultures (Walters and Williams, 1966). When rotated, the cells agglutinated at the normal rate but formed irregular aggregates. On microscopic examination, these aggregates differed from normal in that the cells were in loose contact and there were many intercellular spaces within the clusters.

Suspensions of cells in plasma were treated with cycloheximide (actidione), an inhibitor of protein synthesis, and incubated for 0.5 hour at room temperature. The cells were then mechanically dispersed and placed on the rotary shaker. Normal aggregation took place even in the presence of 200 mg/ml of cycloheximide —a concentration four times that which suppresses 90 per cent of amino acid incorporation in the wing tissue of developing moths (M. J. Hughes, Harvard University, personal communication).

TABLE III

Comparison of the reaggregation of Cynthia cells in plasma derived from Cynthia or from Polyphemus

Proportion of plasma in medium	Reaggregation after 2 hours in:	
	Cynthia plasma	Polyphemus plasma
50%	+++	++(+)
20%	++	++
10%	+	+
0%	0	0

Aggregation-promoting factors in plasma

As summarized in Table I, fat-body cells were suspended in plasma from either diapausing or developing individuals of the same or different species of saturniids. In all cases, the cells reaggregated in typical fashion.

To learn whether plasma is essential for the cohesion of fat-body cells, samples of plasma were diluted serially with Weevers's solution, and an aliquot of cells was suspended in each dilution. The behavior of the suspensions after two hours of rotation is shown in Table II and in Figures 1–4.

In media containing at least 40 per cent plasma the cells underwent maximal aggregation. When the proportion of plasma was reduced to 20 per cent, the degree of agglutination and the size attained by aggregates markedly decreased. This trend continued until in 2.5 per cent plasma no aggregation took place. Similar results were observed when the experiment was repeated using cells and plasma derived from two different species (Table III).

To study the role of the plasma in cellular adhesiveness, an extract was prepared by stirring cell-free hemolymph into 20 volumes of chilled acetone. Approximately 100 mg of dry, yellow precipitate was obtained from each milliliter of plasma. When this powder was dissolved in 2 or 3 volumes of Weevers' solution, the resulting medium sustained normal reaggregation.

Samples of insect hemolymph were dialyzed against 400 volumes of Weevers' saline for 24 hours at 2°–3° C. When assayed, the dialysate was fully effective in promoting aggregation. Likewise, full activity was retained by a Diaflo ultrafiltration membrane (type UM-1; Amicon Corp., Cambridge, Mass.), suggesting for the active factor a molecular weight greater than 10,000 (manufacturer's specification).

The heat stability of the plasma component required for aggregation was investigated by heating samples of whole plasma in a water bath. After being heated to 50°–55° C for ten minutes, plasma sustained reaggregation only slightly less well than unheated plasma. When plasma was heated to 60°–65° C, a fine precipitate formed which did not dissolve upon cooling; the supernatant was assayed and found to be completely inactive.

To determine whether the factor required for aggregation was proteinaceous, 0.2 per cent crystalline trypsin in Weevers's saline was added to an equal volume of plasma to give a final enzyme concentration of 1 mg/ml. After incubation at 25° C for 12 hours, 10 mg/ml of soybean trypsin inhibitor (SBTI [Sigma Chemical Corp., St. Louis]) was added. To additional samples of plasma, trypsin solution and SBTI were simultaneously added; as a further control, an aliquot of plasma was incubated with SBTI in the absence of enzyme. The trypsin digests and the controls were assayed with freshly dissociated fat-body cells. Both controls showed normal reaggregation, whereas the cells suspended in trypsin digest remained completely dispersed.

Solubility of the factor

The foregoing experiments clearly point to one or more plasma proteins as indispensible for the mutual adhesion of fat-body cells. To characterize this factor in further detail, proteins were selectively precipitated from cell-free Polyphemus hemolymph by diluting it with distilled water. After ten-fold dilution, the copious concentrates and the acetone powders were each dissolved in sufficient Weevers' saline. (The material dissolved readily except for a small, gummy fraction which was removed by centrifugation.) This medium, consisting of a saline solution of water-insoluble globulins, was assayed in the usual manner and found to support normal reaggregation.

Two of the aqueous supernatants remaining after precipitation of the globulins were concentrated to the volume of the original plasma by means of the Diaflo ultrafiltration apparatus. Two similiar supernatants were extracted in cold acetone to yield 75 mg of dry precipitate for each milliliter of original plasma. The concentrates and the acetone powders were each dissolved in sufficient Weevers' saline to reconstitute a 50 per cent plasma solution and then assayed. Only a trace of reaggregation could be observed in any of these solutions.

In a similar series of experiments, the globulins were precipitated from Cynthia plasma by twelve-fold dilution with distilled water. The precipitate was dissolved in Weevers' solution and dialyzed against 300 volumes of the same for 24 hours at 2°–3° C. Meanwhile, the aqueous supernatant was concentrated by dialysis in a bath of Carbowax-6000 (Union Carbide Corp., New York) until the volume of the original plasma was restored; it was then dialyzed against 300 volumes of Weevers' solution. Both the globulin and aqueous fractions were adjusted to a volume equivalent to twice that of the original plasma and assayed. Reaggregation was maximal in the suspensions containing the globulin fraction but almost nil in the aqueous fraction.

Diapausing Polyphemus plasma was dialyzed to remove free amino acids, and its protein content was determined by the method of Lowry, Rosebrough, Farr and Randall (1951). Total protein was found to be 85 mg/ml, of which 15 mg/ml precipitated upon ten-fold dilution with water.

Several vertebrate tissue extracts or purified proteins were assayed for their ability to promote the aggregation of fat-body cells. A crude acetone-precipitate of pig liver (Pentex, Inc., Kankakee, Ill.) was dissolved in Weevers' solution in the amount of 30 mg/ml; fat-body cells suspended in this solution failed to reaggregate. Similar negative results were obtained for 30 mg/ml solutions of bovine serum albumin, porcine alpha or beta globulins (Pentex, Inc.), or rabbit gamma globulin (kindly supplied by Professor A. M. Pappenheimer, Jr.).

TABLE IV

Reaggregation of Cynthia fat-body cells suspended in dialyzed plasma

Number of experiments	Dialysis bath	Salt added to dialysate	Aggregation after 2 hours
6	Weevers' soln.	none	+++
6	Ca- & Mg-free soln.	none	0 or (+)
1	Ca- & Mg-free	$CaCl_2$ (4 mM)	+++
1	Ca- & Mg-free	$CaCl_2$ (10 mM)	+++
1	Ca- & Mg-free	$MgCl_2$ (18 mM)	+++
1	Ca- & Mg-free	$MgCl_2$ (10 mM)	++(+)

Role of divalent cations

In stationary cultures of fat-body cells, reaggregation is completely inhibited by the chelating agent EDTA (Walters and Williams, 1966). In the present study, 0.075 *M* EDTA fully inhibited reaggregation in rotating suspensions of fat-body cells. When the concentration of EDTA was reduced to 0.030 *M*, the cells clumped in the normal way. Thus, EDTA is effective only at a concentration equivalent to the combined concentrations of calcium and magnesium ions in saturniid plasma (*cf.* Michejda and Thiers, 1963).

More direct evidence of the participation of calcium and magnesium ions was sought in the following experiments. Precipitated plasma globulins were dissolved in either 0.16 N sodium chloride or a modified Weevers' solution lacking calcium and magnesium. (The latter was prepared by replacing the calcium and magnesium salts with an osmotically equivalent amount of glucose.) Contrary to expectation, reaggregation took place in both media, and only to a slightly lesser degree than in the control medium which contained exogenous calcium and magnesium ions.

This puzzling result suggested that the divalent cations might be bound to the globulins and precipitated with them. Therefore, samples of plasma were subjected to dialysis against 400 volumes of calcium- and magnesium-free saline for 24 hours at 2°–3° C. Following this treatment, the plasma proteins were unable to promote aggregation. The addition of crystals of either calcium chloride or magnesium chloride promptly restored their activity (Table IV).

Discussion

Cellular metabolism and adhesion

Vertebrate embryonic cells are unable to reaggregate when treated with inhibitors of protein and nucleic acid synthesis (Moscona and Moscona, 1963). The same does not seem to be true of many invertebrate cells. Sea urchin and sponge cells continue to reassemble after the suppression of protein synthesis (Guidice, 1965; Humphreys, 1965). The inhibition of general metabolism prevents reaggregation of invertebrate cells only in situations where cell locomotion is necessary to bring the cells into mutual contact (Guidice, 1965; Walters and Williams, 1966).

The present findings indicate that the adhesiveness of fat-body cells, like that of sea urchin and sponge cells, depends neither on concurrent protein synthesis nor on general metabolism. The fact that loosely structured aggregates formed at low temperatures or in the presence of dinitrophenol suggests that reaggregation normally takes place in two steps: an initial loose cohesion followed by the adoption of a "close packing" arrangement. Perhaps the latter process involves a certain degree of active motility on the part of the cells.

It is possible that the technique of dissociation is responsible for the difference in the ability of vertebrate and invertebrate cells to reaggregate in the absence of protein synthesis. Vertebrate embryonic tissues are commonly dissociated with trypsin, a treatment which may denude the cells of certain proteins essential for adhesion. By contrast, the cells of sponges, sea urchins, and insects are separated by ionic or mechanical means and may retain preformed surface materials destroyed by trypsin.

Extrinsic factors determining adhesion

There are two principal requirements for the successful reaggregation of fat-body cells under the experimental conditions described here. First, the medium must contain divalent cations. This requirement has also been recognized in other reaggregating systems (Galtsoff, 1925; Moscona and Moscona, 1952; Humphreys, 1963). Indeed, treatment with chelating agents or immersion in calcium- and magnesium-free media have often been used to dissociate embryonic or even adult tissues (Anderson, 1953; Guidice, 1962; Jones and Elsdale, 1963). The role of calcium has received primary attention. In the case of sponges, however, calcium and magnesium salts have been found interchangeable (Humphreys, 1963), and the same is true of dissociated fat-body cells (Table IV).

In a study of the ionic composition of silkworm plasma, Michejda and Thiers (1963) found unusually high concentrations of divalent cations, especially magnesium. Much lower concentrations are known to be effective in physiological solutions (Weevers, 1966), implying that the ions are extensively bound to other molecules. The presence of divalent cations in the precipitated globulins, as demonstrated in the present study, confirms that significant amounts of these ions are normally bound to plasma proteins.

A second requirement for the reaggregation of fat-body cells is that the medium contain a water-insoluble proteinaceous material present in insect plasma at all stages of development. The requirement is specific in that it was not possible to substitute non-insect proteins (*viz.*, vertebrate liver extract, serum albumin, or alpha, beta, or gamma globulins). It is not species-specific, however, since the hemolymph of different genera of silkworms was fully effective when interchanged (Tables I and III).

Various proteins, exogenous or produced by the cells themselves, have also been implicated in other reaggregating systems. Certain sponge cells secrete a highly specific glycoprotein which is essential to their mutual cohesion (Humphreys, 1967). The dissociated cells of vertebrate embryos reaggregate only in complex media. Evidently, a number of proteinaceous factors must be present, many of which appear to be the product of cellular metabolism (Moscona, 1965). A factor in the supernatant of avian tissue cultures has been recently shown specifically to enhance the aggregation of dissociated cells from the same kind of tissue (Lilien, 1968; Kuroda, 1968). These findings have been interpreted as evidence for specific macromolecules which cement cells together (Moscona, 1968). Other workers question the existence of such binding substances and consider the soluble proteins to serve an ancillary function in adhesion (Steinberg, 1964; Curtis and Greaves, 1965; Jones, 1966; Curtis, 1967).

The function of the divalent cations has also been subject to various interpretations. Among the postulated effects have been the following: (1) formation of intercellular bridges (Steinberg, 1958); (2) stabilization of extracellular cementing material (Chambers and Chambers, 1961); (3) reduction of electrostatic repulsion between apposing membranes (Curtis, 1962); (4) participation in the enzymatic activity of adhesive sites on the cell surface (Jones, 1966). In the case of fat-body cells, present evidence does not allow us to decide among these possibilities. Despite this fact, it is amply clear that cellular adhesiveness requires divalent cations as well as one or more plasma proteins. Evidently, these factors are intimately related, since reassociation of the fat-body cells was sustained by the calcium and magnesium precipitated with the active proteinaceous material.

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Summary

1. Cells dissociated from the fat-body of developing saturniid moths underwent rapid reaggregation when subjected to rotary agitation in blood plasma.

2. The rate and degree of aggregation was unaffected by low temperature or the presence of inhibitors such as dinitrophenol or cycloheximide.

3. Normal aggregation also occurred when plasma from different developmental stages or different silkworm genera was substituted.

4. When the proportion of plasma in the medium was reduced to less than 40 per cent, the degree of aggregation as measured by the size of aggregates progressively declined. No aggregation took place in physiological saline.

5. Cell adhesion was found to depend on a plasma factor which was non-dialyzable, heat-labile, inactivated by trypsin and insoluble in distilled water.

6. Vertebrate proteins (liver extract, serum albumin, or alpha, beta, or gamma globulins) could not be substituted for the plasma factor.

7. Cell adhesion also required the presence of either calcium or magnesium ions. These ions precipitated with the active proteinaceous material in amounts sufficient for reaggregation.

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THE EFFECT OF COLCHICINE ON MIGRATION OF PIGMENT GRANULES IN THE MELANOPHORES OF *FUNDULUS HETEROCLITUS*[1]

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Previous work has shown that colchicine has an effect on the movement of melanin granules in the melanophores of isolated frog skin. Thus pretreatment of frog skin with colchicine enhances the darkening produced by melanocyte-stimulating hormone (MSH) (Malawista, 1965) and inhibits the lightening which follows upon the removal of MSH (Wright, 1955; Malawista, 1965). Colchicine has also been found to inhibit the aggregation of pigment in tissue cultured melanophores of *Xenopus laevis* after removal of MSH (Kulemann, 1960). The proposal has been advanced that in frog melanophores this drug has an effect on cytoplasmic viscosity (Malawista, 1965; Malawista, Asterita, and Marsland, 1966).

Recently Bikle, Tilney and Porter (1966) have shown that an ordered array of microtubules is present in the branching processes of the melanophores of the killifish, *Fundulus heteroclitus*. These observations have been confirmed by Novales and Novales (1966) and Green (1968). Bikle *et al.* (1966) suggested that these microtubules may be involved in the movement of the pigment granules.

Colchicine has been demonstrated to have an effect on cytoplasmic microtubules. This alkaloid brings about disruption of mitotic spindle microtubules in a variety of dividing cells (de Harven and Bernhard, 1956; Robbins and Gonatas, 1964; Pickett-Heaps, 1967). Colchicine also brings about disassembly of microtubules in a number of interphase cells. These include HeLa cells (Robbins and Gonatas, 1964; Freed, Bhisey, and Lebowitz, 1968), axopods of *Actinosphaerium* (Tilney, 1965), human lymphocytes (Malawista and Bensch, 1967), and human platelets (White, 1968). The mechanism by which colchicine breaks down microtubules is unknown. The effect of this drug on the mitotic apparatus has been attributed to a possible combination with sulfhydryl groups of the spindle proteins (Dustin, 1949; Galzigna, 1961). Reaction of colchicine with thiol groups is also indicated by studies on chick embryos in which the inhibitory effect of colchicine on embryonic induction is completely reversed by cysteine hydrochloride (Diwan, 1966). In view of the known effect of colchicine on microtubules, a study was undertaken to test the effects of this drug on pigment migration in *Fundulus* melanophores. In addition, in order to gain further insight into the mechanism of action of colchicine, work was carried out to see if its effect could be reversed by cysteine hydrochloride. The action of colchicine was studied in relation to its effect on the response of the melanophores to epinephrine. Epinephrine was used because it produces rapid aggregation of the melanin in *Fundulus* melanophores (Spaeth and Barbour, 1917) and has been shown to act directly on the fish melanophore (Fujii, 1961).

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MATERIALS AND METHODS

Experiments were performed with isolated scales from mature specimens of the killifish, *Fundulus heteroclitus.* The fishes were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts. They were kept in an "Instant Ocean" Culture System in artificial sea water which was maintained between 10 and 13° C. For each experiment scales were removed with fine forceps from the anterior dorsal and dorsolateral regions of the fish. The scales were mounted on a glass coverslip which was sealed to a Dick perfusion chamber (Dick, 1955). The scales were mounted and the chamber was sealed with Vaspar (4 parts beeswax to 1 part yellow vaseline). Ringer's solution was perfused through the chamber.

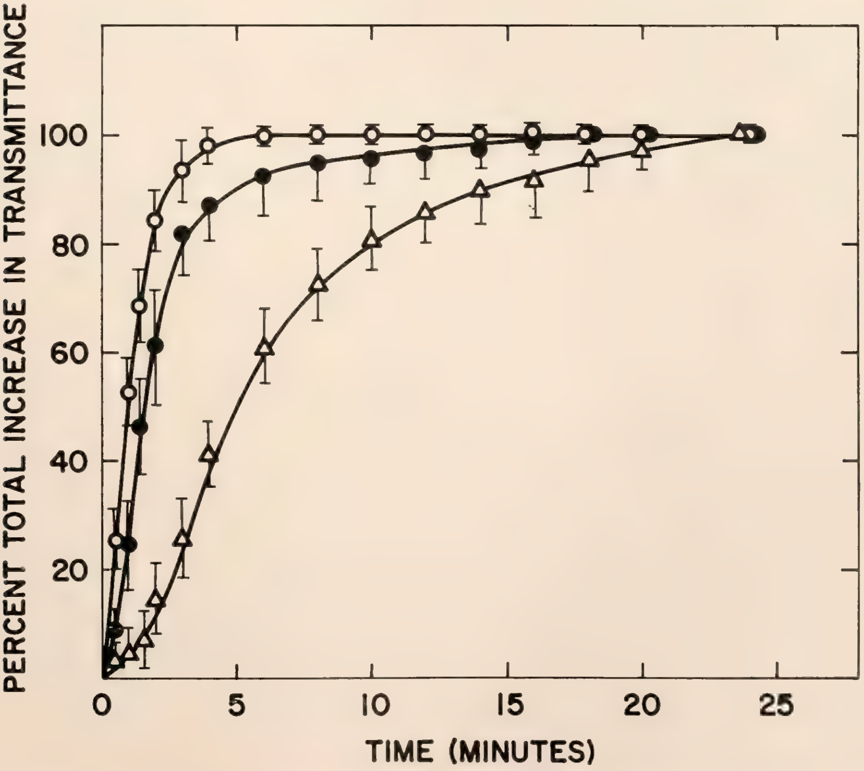

FIGURE 1. The effects of colchicine pretreatment for 40 minutes on the response of melanophores to 0.06 m*M* epinphrine. Points are means ± standard errors. (○) Ringer, 18 scales; (●) 0.5 m*M* colchicine, 8 scales; (△) 5 m*M* colchicine, 10 scales.

A scale remained in Ringer until the melanin granules in the melanophores were dispersed (15 to 30 minutes). The test solutions were then perfused through the chamber.

All test substances were freshly prepared for each experiment. Each was dissolved in frog Ringer's solution, which had the following composition in g per l of distilled water: NaCl, 6.50; KCl, 0.14; $CaCl_2 \cdot 2H_2O$, 0.16; $NaHCO_3$, 0.20. The pH of the solutions was between 7.0 and 7.4. Colchicine (Nutritional Biochemicals Corp.) was prepared at 5 m*M* and 0.5 m*M* concentrations and protected from light before use. A 5 m*M* and 50 m*M* solution of cysteine hydrochloride (Nutritional Biochemicals Corp.) was made up in Ringer buffered, respectively, at pH 7.4 with 5 m*M* and 10 m*M* Tris-HCl buffer. The pH of both solutions was adjusted back to neutrality with a few drops of 0.10 N NaOH. Epinephrine was prepared by dilution of 1:1000 adrenalin chloride (Parke, Davis and Co.) with Ringer to give a 0.06 m*M* solution. The experiments were run at room temperature, between 22 and 24° C.

Changes in the state of melanin dispersion in the melanophores were measured by a photoelectric method (Hill, Parkinson and Solandt, 1935; Smith, 1936; Fujii, 1959). Light transmitted through the scale was directed onto the search unit of a Photovolt Multiplier Photometer, model 520-M. This unit replaced one ocular of a Spencer binocular light microscope. Aggregation of the pigment was measured as an increase in per cent transmittance. The response of the melanophores to a mixture of colchicine and epinephrine was first studied, after which the effect of pretreatment in colchicine on the response to epinephrine was tested. The effect of colchicine on punctate epinephrine-treated melanophores was also studied. The degree of expansion was measured in ocular micrometer units. This expansion, expressed as a percentage of the original response, was based on the difference between the diameter of the melanophore in the fully aggregated state and the greatest diameter in the fully dispersed condition. The ability of cysteine hydrochloride to reverse the effect of colchicine was finally tested.

RESULTS

A 0.06 m*M* solution of epinephrine was found to be the minimum concentration which produced consistent rapid aggregation of the melanin. With a mixture of colchicine and epinephrine, only a slight decrease in the rate of aggregation of the pigment occurred as compared to a combination of Ringer and epinephrine. Thus, pretreatment in colchicine was carried out. Pretreatment produced a decrease in the rate of melanin aggregation in response to epinephrine as compared to the untreated scales. The rate of response depended upon the length of pretreatment and the concentration of colchicine. Thus 40 minutes pretreatment in 0.5 m*M* colchicine had only a slight effect on the rate of pigment aggregation (Fig. 1). However, 5 m*M* colchicine had a significant effect (Fig. 1). By the end of 6 minutes the difference in per cent aggregation was significant at the 5% level. When the pretreatment time was increased to 90 minutes, the response to epinephrine was greatly decreased (Fig. 2). Pigment in melanophores pretreated in Ringer was aggregated within 6 minutes, whereas in those pretreated with colchicine the pigment took 20 to 30 minutes to aggregate. The effects of colchicine could not be "washed out," as shown by the following. Five scales were pretreated

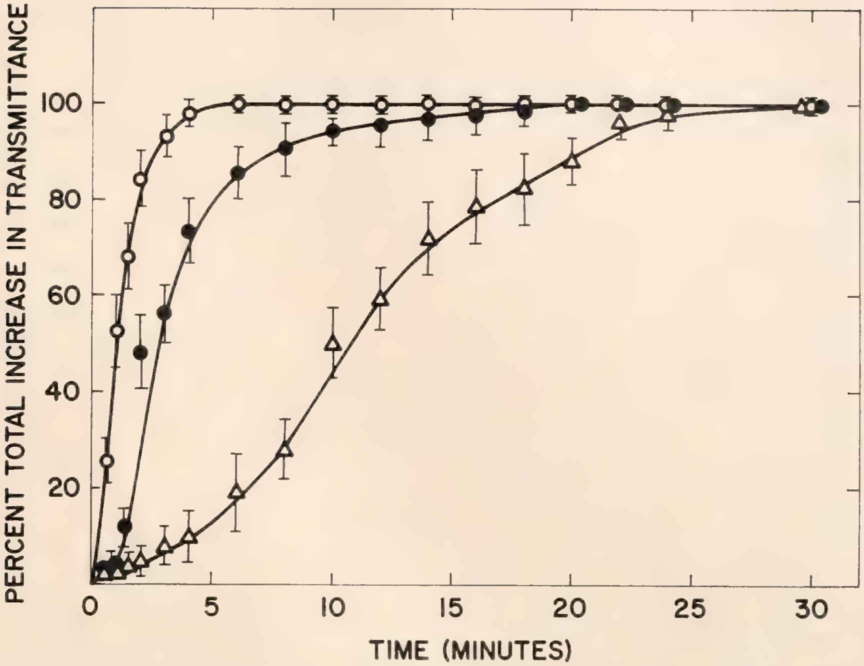

FIGURE 2. The effects of colchicine pretreatment for 90 minutes on the response of melanophores to 0.06 m*M* epinephrine. Points are means ± standard errors. (○) Ringer, 18 scales; (●) 0.5 m*M* colchicine, 8 scales; (△) 5 m*M* colchicine, 10 scales.

in 5 m*M* colchicine for 60 minutes. The scales were then perfused with several changes of Ringer for 60 minutes. When epinephrine was added the results were the same as those obtained from the unwashed scales.

Colchicine also produced dispersion of pigment in punctate epinephrine-treated melanophores. A 5 m*M* solution of colchicine brought about a dispersion of pigment granules which was significantly greater than that produced by Ringer alone (Fig. 3). By the end of 60 minutes the difference was significant at the 5% level. Enhanced dispersion was observed after 80 minutes treatment in colchicine.

Incubation in 50 m*M* cysteine hydrochloride alone following a 60 minute pretreatment with 5 m*M* colchicine had no effect on the melanophores. However, the effects of colchicine on the response to epinephrine were reversed by cysteine (Fig. 4). This reversal depended upon the concentration of cysteine used. Thus, 5 m*M* cysteine did not have a significant effect on colchicine action, whereas, treatment in 50 m*M* cysteine resulted in a normal response to epinephrine. Cysteine by itself had no effect on dispersion of pigment. A 50 m*M* solution had the same effect as Ringer on punctate epinephrine-treated melanophores. Pretreatment with cysteine also did not affect dispersion produced by colchicine. Increasing the pretreatment in 10 m*M* cysteine from 10 to 20 minutes produced only a slight decrease in the dispersion response. Increasing the concentration of cysteine to 50 m*M* slightly enhanced the initial dispersion produced by colchicine.

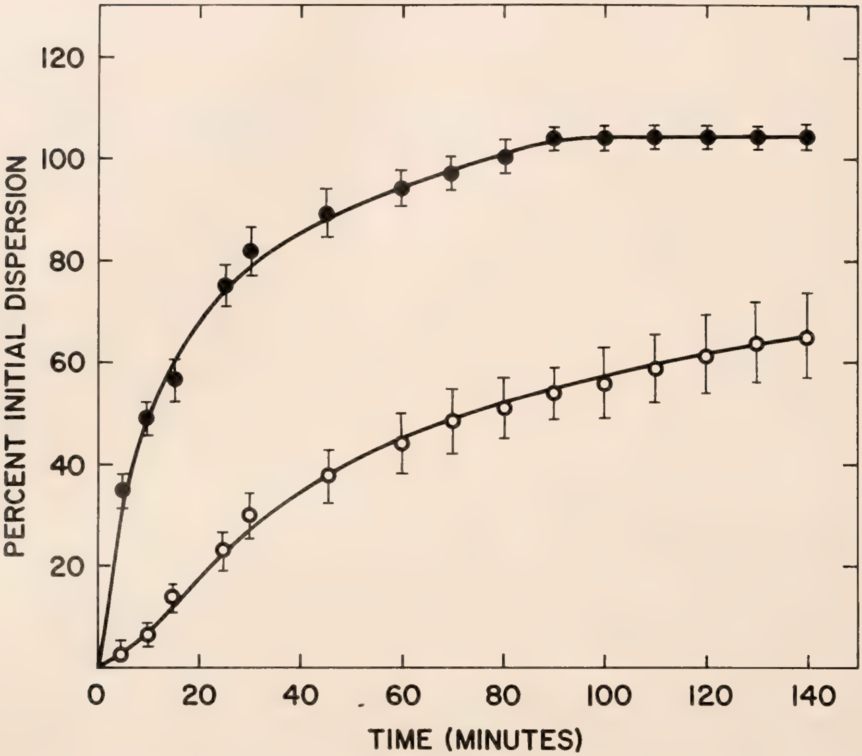

FIGURE 3. Response of punctate epinephrine-treated melanophores to colchicine. Points are means ± standard errors. (○) Ringer, 24 scales; (●) 5 mM colchichine, 21 scales.

DISCUSSION

Colchicine decreased the rate of pigment aggregation in *Fundulus* melanophores in response to epinephrine. The action of this drug depended upon its concentration and pretreatment time (Figs. 1 and 2). The concentrations of colchicine used here are within the ranges that have been used on various cell types without affecting their viability. These include cultured fibroblasts from chick embryo heart (Miszurski, 1949), onion root tip (Sedar and Wilson, 1951) and sea urchin gastrulae (Tilney and Gibbons, 1968). In the present study, the effects produced by colchicine are similar to those observed by Malawista (1965) on dermal melanophores in frog skin. Malawista found that colchicine produced partial inhibition of lightening in skins in response to epinephrine. In the present work, colchicine also brought about dispersion of pigment granules in punctate melanophores which was greater than that produced by Ringer's solution alone. Again, this is in accord with Malawista's results wherein he observed that colchicine produced a darkening of lightened frog skins.

There are several possibilities as to the mode of action of colchicine. This alkaloid might affect the viscosity of the cytoplasm bringing about solation and inhibiting the gelation which is necessary for or which accompanies aggregation of

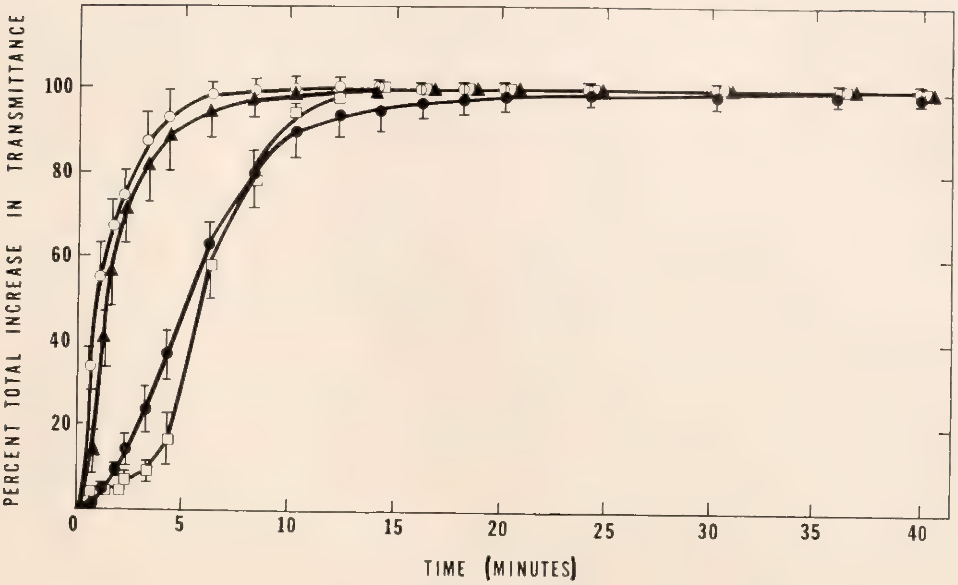

FIGURE 4. Effects of cysteine hydrochloride on the response of colchicine-treated melanophores to 0.06 m*M* epinephrine. Points are means ± standard errors. Pretreatment for 60 minutes in (○) Ringer, 16 scales; (●) 5 m*M* colchicine, 12 scales; (□) 5 m*M* colchicine plus 30 minutes in 5 m*M* cysteine, 5 scales; (▲) 5 m*M* colchicine plus 30 minutes in 50 m*M* cysteine, 13 scales.

the pigment. Matthews (1931) and Marsland (1944) have presented evidence for the view that cytoplasmic solation occurs during melanin dispersion and that melanin aggregation is associated with gelation. Colchicine is known to decrease cytoplasmic viscosity in sea urchin eggs (Beams and Evans, 1940), grasshopper neuroblasts (Gaulden and Carlson, 1951), tissue cultured rat striated muscle fibers (Godman and Murray, 1953; Godman, 1955) and cultured rat fibroblasts (Murray, deLam, and Chargaff, 1955). Malawista (1965) has proposed a general model of colchicine action on gelated cytoplasmic systems. In this model, colchicine, in a gradual, dosage-dependent manner lowers the potential limit of gelation and thus interferes with the maintenance of the gelated state. It is thus possible that in *Fundulus* melanophores colchicine has some effect on the sol-to-gel equilibrium, interfering with the development or maintenance of the gelated condition.

The action of colchicine has also been attributed to a disassembly of cytoplasmic microtubules. This alkaloid appears to disrupt the microtubules by binding to sites of interaction between the protein subunits (Taylor, 1965; Borisy and Taylor, 1967a, 1967b; Shelanski and Taylor, 1967). Dissolution of microtubules by colchicine has been found to be correlated with cessation of particle movement. Tilney (1965) observed that under the influence of colchicine particle streaming within the axopods of a heliozian ceases and coincidently the microtubules disassemble. Freed (1965) also found that treatment of HeLa cells with colchicine led to an inhibition of particle movement and a disappearance of microtubules (Freed, Bhisey, and Lebowitz, 1968). In *Fundulus* melanophores, microtubules run parallel to the longitudinal axis of the cell processes (Bikle, Tilney, and Porter, 1966; Green, 1968). The melanin granules in these extensions are aligned in columns and appear to move parallel to the microtubules along relatively fixed channels. As in other cell types these microtubules are arranged in such a way as to give support and thus serve as cytoskeletal elements, to guide intracellular migration of cytoplasmic particles, and to possibly exert a motive force on particle movement (Sandborn, Koen, McNabb, and Moore, 1964). However, the precise role of these tubular structures is unknown. It is possible that in *Fundulus* melanophores colchicine is acting upon the microtubules and in turn influencing pigment migration. Since colchicine decreases the rate of pigment aggregation but not dispersion, this indicates that microtubules, if involved, influence only the aggregation of the pigment granules.

The effects of colchicine are similar to those produced by high hydrostatic pressure. High hydrostatic pressure produces dispersion and inhibits aggregation of pigment granules in *Fundulus* melanophores (Marsland, 1944; Marsland and Meisner, 1967). It also causes breakdown of the microtubules of the mitotic apparatus (Zimmerman and Marsland, 1964) and of heliozoan axopodia (Tilney, Hiramoto, and Marsland, 1966). This evidence thus indicates that colchicine and high hydrostatic pressure produce similar changes. However, it is unknown whether these agents produce their effect by acting on cytoplasmic viscosity, microtubules, or both.

Heavy water (D_2O) antagonizes the effect of high hydrostatic pressure on *Fundulus* melanophores, possibly through stabilization of microtubules or cytoplasmic gelation (Marsland and Meisner, 1967). The effects of colchicine are opposite to those produced by D_2O. Studies indicate that D_2O stabilizes the microtubules of the mitotic appartus (Marsland and Zimmerman, 1965) and of heliozoan axopodia (Marsland and Hirshfield, 1968), whereas colchicine breaks down these structures. Low concentrations of D_2O oppose the anti-mitotic effects of colchicine on the mitotic spindle of sea urchin eggs (Marsland and Hecht, 1968). Thus it would be of interest to see if D_2O opposes the action of colchicine on melanophores, in particular, the action of colchicine on the response to epinephrine.

It is also possible that colchicine could be interfering in some manner in one of the steps coupling epinephrine action to melanin aggregation, perhaps at the level of the epinephrine receptors. However, there is no evidence for this view or reason to believe that it is acting in this manner.

Cysteine hydrochloride by itself had no direct effect on pigment migration. It did not produce aggregation of pigment of colchicine-treated melanophores nor did it increase the rate of dispersion beyond that produced by Ringer, in punctate melanophores. Shizume, Lerner and Fitzpatrick (1954) also found that cysteine had no direct effect on frog melanophores. However, cysteine reverses the effect of colchicine on the response of melanophores to epinephrine (Fig. 4). This indicates that the action of colchicine involves a reaction with sulfhydryl groups. Work in other systems points to a similar action for colchicine. For example, the inhibitory effect of colchicine on induction in chick embryos is also reversed by cysteine hydrochloride, thus suggesting a reaction of colchicine with sulfhydryl groups (Diwan, 1966). Work on the mitotic spindle also indicates that colchicine may exert its antimitotic action by reversible oxidation of thiolic proteins (Dustin, 1949;

Galzigna, 1961). On the other hand, the dispersing effect of colchicine was not reversed by cysteine. This may indicate that the action of colchicine on dispersion does not depend on a tying up of SH groups.

SUMMARY

1. The effects of colchicine were studied on pigment migration in melanophores of the killifish, *Fundulus heteroclitus.*

2. Pretreatment of the melanophores in colchicine resulted in a decrease in the rate of melanin aggregation in response to epinephrine. The rate of response depended upon the length of pretreatment and the concentration of colchicine.

3. Colchicine produced dispersion of pigment in punctate epinephrine-treated melanophores which was greater than that produced by Ringer alone.

4. Cysteine reversed the effect of colchicine on the response of melanophores to epinephrine. This reversal depended upon the concentration of cysteine used.

5. The mechanism of action of colchicine is unclear but is discussed in relation to possible effects on cytoplasmic viscosity and microtubules.

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CONTINUOUS AND RHYTHMIC REPRODUCTIVE CYCLE OBSERVED IN *PERIPLANETA AMERICANA* (L.)

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The secretion of vitellogenic proteins (vitellogenins) by the fat body into the blood and their sequestration by the oocytes have been found in the reproductive cycles of *Periplaneta americana* (Mills, Greenslade and Couch, 1966; Adiyodi and Nayar, 1967; Nielsen and Mills, 1968), *Leucophaea maderae* (Engelmann and Penney, 1966) and *Nauphoeta cinerea* (Adiyodi, 1967). Because these cycles differ in many respects, including their duration, there are basic differences in the schedule of vitellogenin secretion and yolk formation.

The purpose of this communication is to elucidate the reproductive functions of blood proteins in *Periplaneta americana*, including its two vitellogenins, by correlating their fluctuations with cyclic changes in the rate of yolk deposition and with other phases of reproduction. Precise information on these relationships from a number of species with varying cycle characteristics should provide better insight into the cellular processes and the control mechanisms which govern reproduction.

METHODS AND MATERIALS

Colonies of *Periplaneta americana* were fed Purina laboratory chow and water, and maintained at 26° C, 70% relative humidity, with a 12:12 hour photoperiod in a constant temperature incubator. Adult females were staged according to the time after the ootheca begins to form (BF); the time of ootheca formation was designated as day 1.

Staged females were selected every 24 hours after ootheca formation. Samples of blood were collected according to the method of Mills *et al.* (1966), and the ovaries were dissected out in Ringers solution (van Asparen and van Esch, 1956). The length and width of the basal and penultimate oocytes were measured and the volume was calculated using the formula for a prolate spheroid,

$$V = (W/2)^2(L/2)(4/3\,\pi).$$

Yolk proteins were obtained either by homogenizing oocytes removed from the ovaries or by collecting the contents of newly formed oothecae. Ten or twenty oocytes were homogenized in 0.4 M NaCl buffered at pH 7.2 and the mixture was centrifuged at 10,000 g for 30 minutes in the cold. A lipid cap rested on the clear supernatant and a sediment of membrane and ovarian sheath formed a pellet at the bottom of the tube. When oocytes were broken in 0.4 M NaCl and

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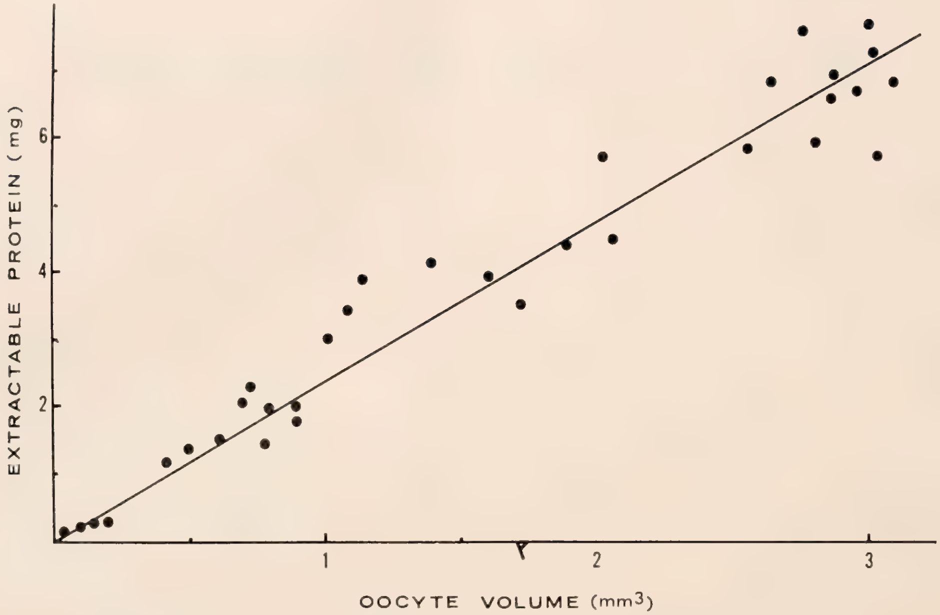

FIGURE 1. Protein content compared with volume in vitellogenic oocytes. Each point is the average of 10 oocytes.

the behavior of yolk fluid observed under low power microscopy, the viscous protein fluid released from broken yolk spheres became soluble; when broken in 0.15 *M* NaCl, precipitation was noted. Thus the necessity of high ionic strength for oocyte protein solubility, reported by Dejmal and Brookes (1968) for *Leucophaea maderae,* was also true for the oocyte proteins of *Periplaneta.* Yolk fluid squeezed from newly formed oothecae, however, differed in this regard. The yolk fluid was centrifuged as above and the protein fraction was removed and a serial dilution was performed in 0.15 *M* NaCl. Oocyte proteins failed to precipitate when diluted to 5% ; below this critical point of dilution, the proteins were precipitated.

Cockroach blood and the supernatant resulting from the centrifugation of homogenized oocytes were measured for total protein concentration using the microbiuret method of Itzhaki and Gill (1964). Vitellogenin concentration was measured by the quantitative immunodiffusion technique (Oudin, 1948) utilizing an antiserum prepared against the yolk protein fraction from newly formed oothecae (Bell, 1969a). The antiserum, solidified with agar in glass tubes, was overlayered with blood or yolk fluid and the rates of migration of the zones of precipitation were recorded. The concentration of the two vitellogenins in female blood, relative to their concentrations in yolk fluid, was calculated by referring to a standard curve of the log of yolk fluid antigen concentration plotted against the rate of migration.

Blood volume determinations were made using the C^{14}-inulin method described by Wharton, Wharton and Lola (1965).

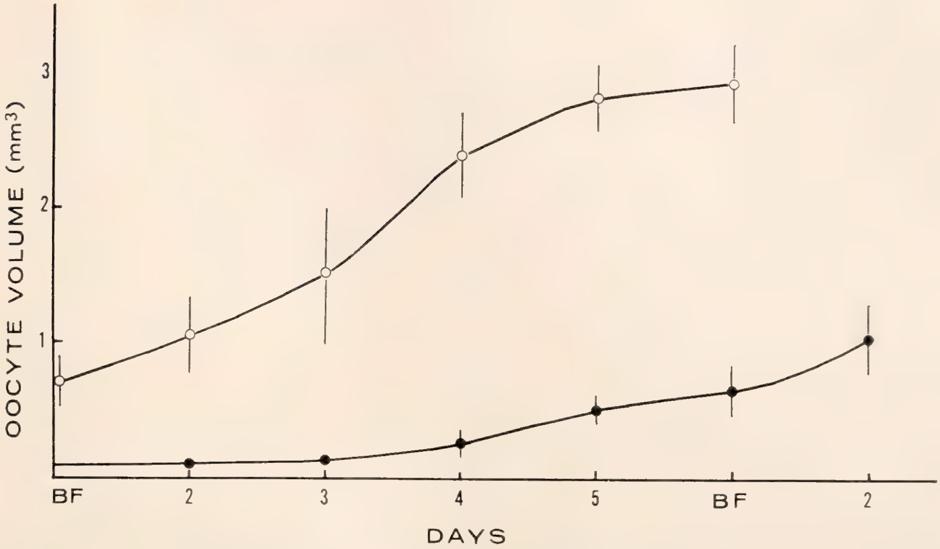

FIGURE 2. Oocyte volume during the 5-day cycle. Each point is the average of 25 to 50 females. Vertical lines are standard deviations [basal oocytes ○, penultimate oocytes ●].

RESULTS

Changes in oocyte volume and protein content during the vitellogenic cycle

The volume of the oocytes increased proportionately with the increase in extractable yolk protein (Fig. 1). The total protein concentration of yolk fluid ob-

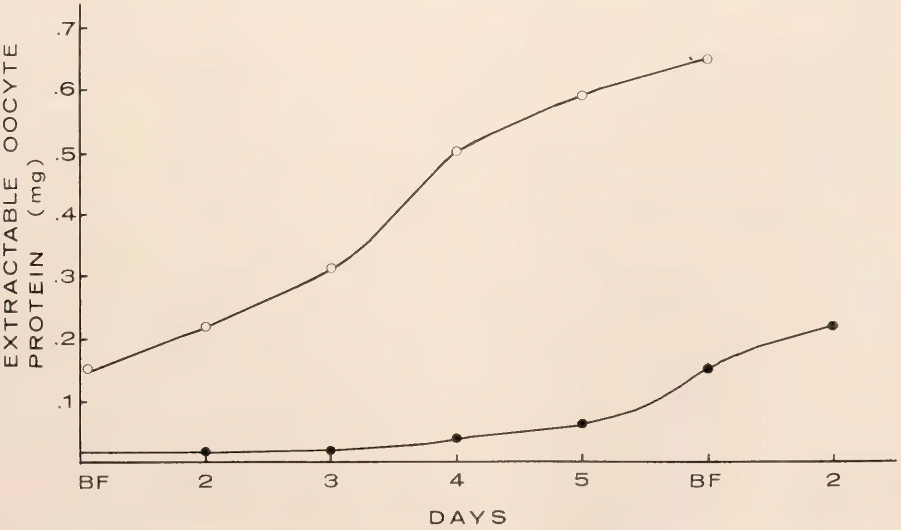

FIGURE 3. Oocyte protein content during the 5-day cycle. Each point is the average of 25 to 50 females [basal oocytes ○, penultimate oocytes ●].

TABLE I

Rate of protein accumulation by oocytes during the 5-day cycle

Time interval (days)	Protein increase (μg/oocyte)			Oocyte protein increase per female (μg)
	Basal	Penultimate	Total	
BF–2	86	8	94	1504
2–3	94	12	106	1690
3–4	160	25	185	2960
4–5	74	21	95	1520
5–BF	43	43	86	1344

tained from oothecae was 225 μg/μl, 88% of which is attributed to vitellogenins A and B (Bell, 1969a), indicating a yolk fluid vitellogenin concentration of 198 μg/μl.

The vitellogenic cycle was found to be a 5-day succession in which an ootheca was formed every fifth day. By the time the basal oocytes had completed yolk formation and were involved in chorion deposition, the penultimate oocytes had reached an advanced stage in yolk formation and continued during and after the basal oocytes were ovulated; thus two oocytes in each ovariole were supporting yolk deposition simultaneously (Roth, 1968). This relationship was observed in both the volume (Fig. 2) and the protein content (Fig. 3) of the oocytes. The continuum is not regular, however, as the amount of protein accumulated by the combined effort of the basal and penultimate oocytes (Table I) differed from one stage in the cycle to another. There was a peak of yolk formation between days 3 and 4, whereas at either end of the cycle the rate was about half that at the peak.

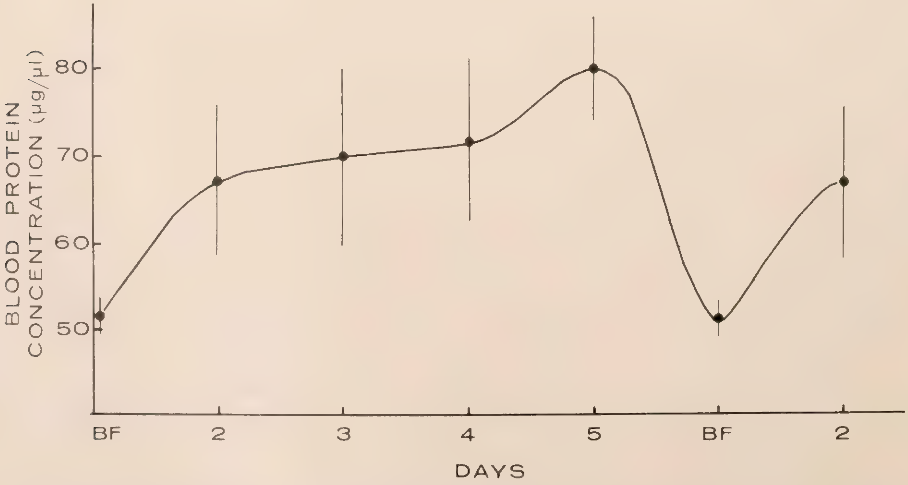

FIGURE 4. Total blood protein concentration during the 5-day cycle. Each point is the average of 20 females. Vertical lines are standard deviations.

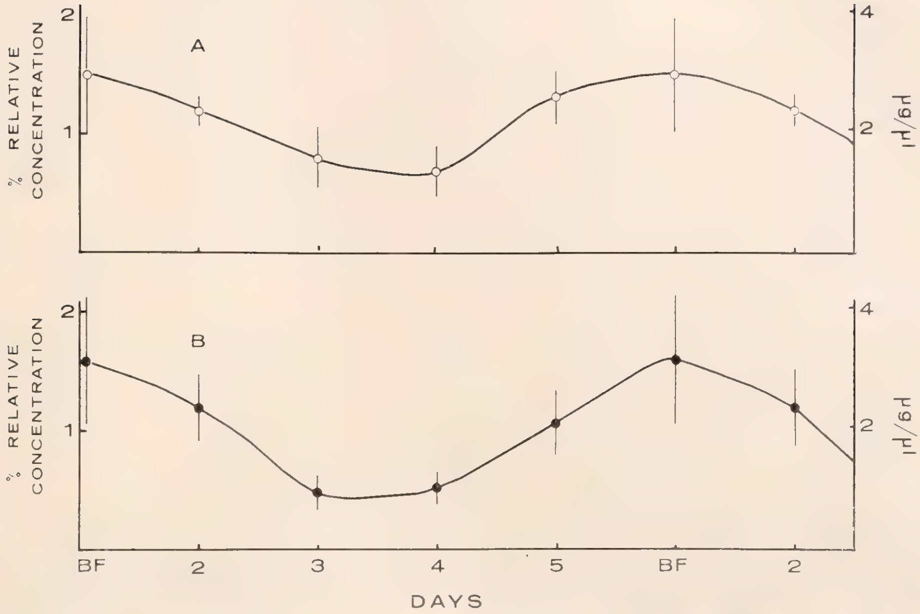

FIGURE 5. Blood vitellogenin concentration during the 5-day cycle. Each point is the average of 20 females. Vertical lines are standard deviations. Per cent concentration is relative to the concentration in the yolk fluid standard, left; estimated protein concentration in µg/µl, right; vitellogenin A ○, vitellogenin B ●.

Changes in blood protein and vitellogenin concentration during the vitellogenic cycle

During the 5-day cycle the total blood protein concentration (Fig. 4) increased gradually to 80 µg/µl on day 5 and then decreased abruptly to 52 µg/µl while the ootheca was being formed. The levels of vitellogenins A and B, which oscillated synchronously (Fig. 5), reached a peak during ootheca formation and declined through days 3 and 4 of the cycle. Vitellogenin concentration in the blood was therefore, as might be expected, lowest when the rate of yolk formation was highest. Total blood protein, on the other hand, while undergoing a 5-day cycle, dropped most precipitously on days 5 to BF when the rate of yolk formation was minimal. When the per cent vitellogenin figures (resulting from Oudin measurements) were converted into µg/µl by referring to the total protein concentration of the yolk fluid standard (225 µg/µl), the combined vitellogenin concentration (of antigens A and B) was equivalent to only 6.5 µg/µl at ootheca formation and 2.0 µg/µl during days 3 and 4. The changes in vitellogenin concentration are therefore slight compared with the 30 µg oscillation in total blood protein observed during the cycle.

To determine whether the fluctuations in protein concentration might be due to changes in the blood volume, the average blood volume for females during each stage of the cycle was measured. The results, depicted in Figure 6, show that the blood volume gradually increased to 207 µl at day 4 and then dropped

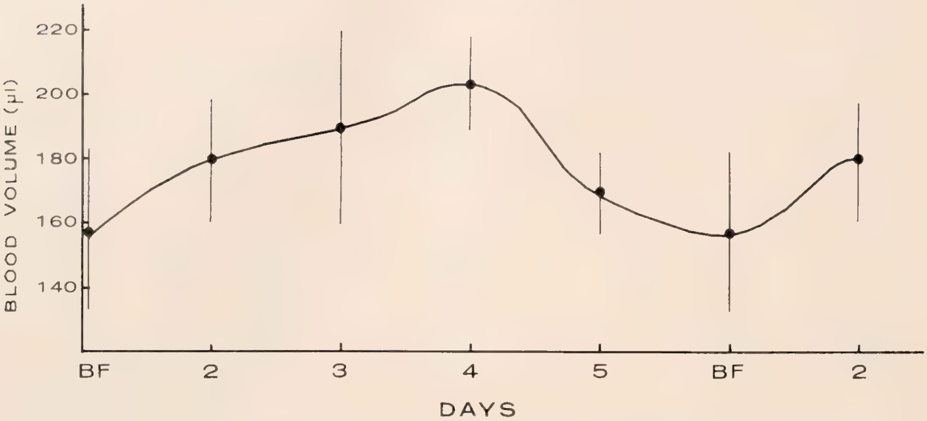

FIGURE 6. Blood volume during the 5-day cycle. Each point is the average of 10 females. Vertical lines are standard deviations.

to 157 μl at the time of ootheca formation. A better indication of the correlation between yolk formation and blood protein content was obtained by multiplying the protein concentration (mg/μl) by the blood volume (μl) to obtain an estimate of the total amount of protein in the blood (Fig. 7). The results indicate that changes in blood volume do not explain the blood protein concentration changes shown in Figure 4, but, on the contrary, amplify the drop in protein correlated with ootheca formation.

The changes in the total blood protein must be due to changes in the relative concentration of non-vitellogenic blood proteins which do not have homologous antibodies to the yolk proteins in the antiserum utilized in this study. That non-vitellogenic blood proteins undergo alterations in relative concentration in female *Periplaneta* is also suggested by electrophoretic analyses of blood proteins during the vitellogenic cycle (Nielsen and Mills, 1968; Adiyodi and Nayar, 1967).

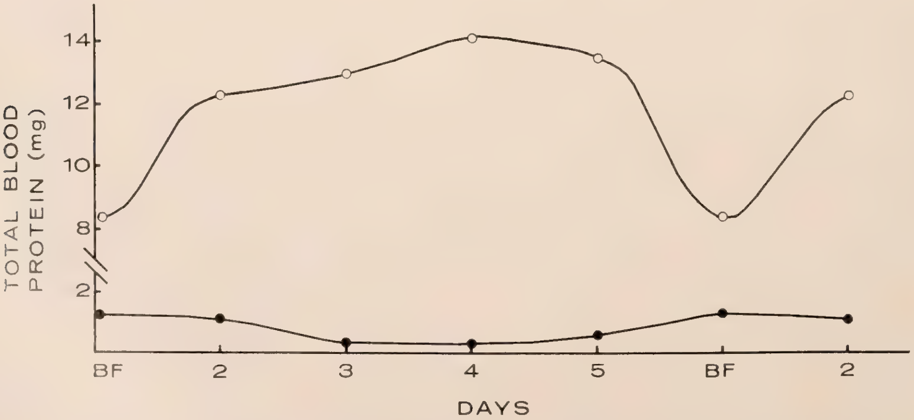

FIGURE 7. Estimation of blood protein content during the 5-day cycle. Total protein is designated as ○, vitellogenin A and B combined as ●.

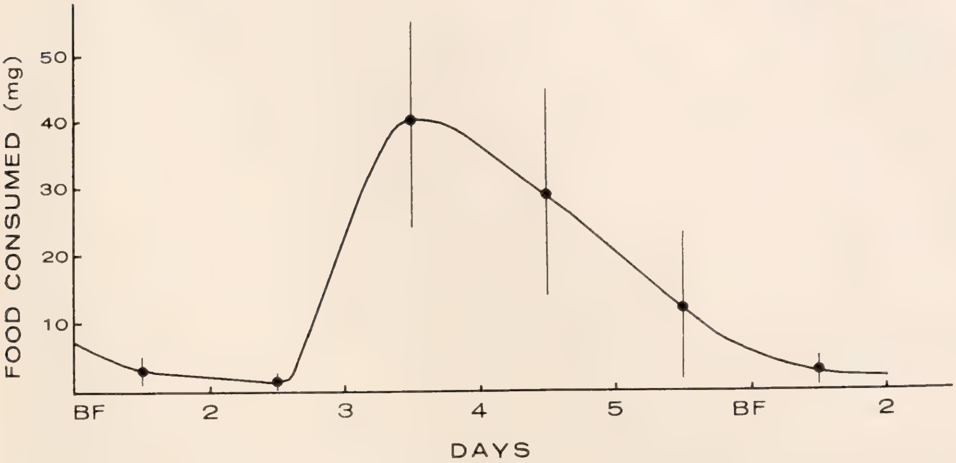

FIGURE 8. Food consumption during the 5-day cycle. Each point is the average of 30 females. Vertical lines are standard deviations.

A similar treatment of the total vitellogenin content of the blood (Fig. 7, solid circles) dampens the fluctuations observed in Figure 5, but the effects of yolk deposition remain apparent. Thus the vitellogenin content of the blood is lowest on days 3 and 4, during the period when yolk deposition is most rapid (Table I), and substantially higher during periods of reduced yolk deposition. That there should be such an effect is not surprising, considering the fact that 9 mg of vitellogenins A and B combined are incorporated into the oocytes during one ovarian cycle, whereas the blood contains at any one time no more than 1.0 mg.

A final indication of cyclicity correlated with ovarian development was observed in the feeding cycle of reproductive females (Fig. 8). The amount of food consumed by vitellogenic females was estimated by weighing the food pellets daily for 30 animals over a 60 day period. There is much variation between individuals, but a hiatus in feeding is evident at the time of ootheca formation, whereas a peak in feeding is observed during the nights of stages 3 and 4. The correlation of food intake with the 5-day cycle might have been expected to provide an explanation for the rise in blood protein content during the first two days after ootheca formation; feeding however, is not initiated until the rise in blood protein is nearly completed. This result necessitates the prediction that there is a cycle in intracellular protein storage in tissues such as the fat body (Dr. Barbara Stay, Department of Zoology, University of Iowa in preparation).

DISCUSSION

There is a wide range of reproductive diversity in cockroaches, extending from oviparity (*Periplaneta americana*), and ovoviviparity (*Leucophaea maderae, Nauphoeta cinerea*) to a resemblance of viviparity (*Diploptera punctata*) (Roth and Willis, 1958). This closely related group of insects thus offers various types of reproductive cycles for study. All cycles which have been adequately investigated seem, nevertheless, to entail as one component the secretion of vitellogenin by the fat body (Pan, Bell and Telfer, 1969) and its sequestration by the oocytes (Adiyodi, 1967; Adiyodi and Nayar, 1967; Nielsen and Mills, 1968; Engelmann and Penney, 1966).

The cycles of the ovoviviparous cockroaches differ in several important respects from that described here for *Periplaneta.* Roth and Stay (1961, 1962), Roth (1964) and Engelmann (1960) have shown that during pregnancy in *Leucophaea* and in *Nauphoeta,* yolk formation may be blocked by a negative feedback from the brood sac which inhibits secretion of the *corpus allatum* gland hormone. Yolk deposition resumes after parturition in *Leucophaea* and begins during late pregnancy in *Nauphoeta.* Engelmann and Penney (1966) have shown that in *Leucophaea* the vitellogenin level in the blood falls to a low value at ovulation, remains undetectable during pregnancy, but rises again at parturition. The total blood protein concentration reflects the falling vitellogenin level at ovulation (6 g/100 g of blood), but does not reflect the absence of vitellogenin during pregnancy (10 g/100 g of blood). In *Nauphoeta,* Adiyodi (1967) described two vitellogenins (electrophoretic fractions 4 and 5) which decrease in concentration relative to other blood proteins during yolk formation, comprise less than 10% of the total blood protein complement at ovulation, and then increase gradually, especially during middle and late pregnancy, to a 20 and 45% of the total blood protein complement.

Two interesting points emerge from these studies. First, during pregnancy, inhibition of the *corpus allatum,* promoted apparently through the proprioception of egg incubation (Roth and Stay, 1961) or humoral ovarian factors (Engelmann, 1964), recedes gradually toward the end of pregnancy (in *Nauphoeta* and *Diploptera*) allowing juvenile hormone secretion. One of the functions of juvenile hormone is to promote the synthesis of vitellogenin (Coles, 1964; Minks, 1967; Thomas and Nation, 1966; Engelmann and Penney, 1966; Bell 1969b), and this presumably accounts for the increase in blood vitellogenin and the initiation of yolk formation during late pregnancy in *Nauphoeta.* In *Leucophaea,* yolk deposition in the next batch of oocytes fails to occur until parturition, and this correlates with the absence of vitellogenin in the blood of pregnant females. Secondly, in the two ovoviviparous species studied, the changes in total blood protein concentration reflect the changes in vitellogenin levels to a different degree. In *Leucophaea* the total blood protein level appears not to correlate with the vitellogenin level, while in Nauphoeta the vitellogenins occupy a large percentage of the total blood protein and seem to be responsible for changes in the total blood protein levels. Because there is a close correlation between yolk formation and depletion of total blood protein resources in *Pyrrhocoris apterus* (Sláma, 1964) and in *Schistocerca gregaria* (Hill, 1962) it is probable that the vitellogenins occupy a large part of the blood protein in these species also.

Reproduction in *Periplaneta* differs from that in ovoviviparous cockroaches in that the eggs are not incubated internally, and the ovarioles contain two oocytes (basal and penultimate) in zone V which are simultaneously forming yolk (Bonhag, 1959; Roth, 1968). Moreover, vitellogenesis is continuous in the American cockroach, although cyclic variability is observed in the rate of this process (Table I). In contrast to the accumulation and depletion of vitellogenins, as in *Leucophaea* and *Nauphoeta,* the vitellogenic proteins of *Periplaneta* do not accumulate in the blood and comprise only about 4% of the total blood protein (Bell, 1969a). Nor are the blood vitellogenins significantly depleted during yolk formation; instead, their concentrations in the blood remain relatively constant, with at most a 50% concentration change correlated with fluctuations in the rate of yolk formation. It appears likely that vitellogenins A and B, being selectively incorporated by the oocytes (Bell, 1969a) are sequestered at a rate equal to their rate of secretion by the fat body.

Total blood protein levels in the American cockroach, as depicted in this paper and by Mills *et al.* (1966), are not obviously related to the vitellogenic cycle. The concentration changes observed are nearly the same in both studies, although different assay methods were used (R. R. Mills, personal communication), and must eventually be related to the schedule of blood protein synthesis, feeding activity, and to blood protein utilization. Changes in blood volume appear not to cause the observed fluctuations in total blood protein concentration. As predicted from studies on diuretic and antidiuretic hormone titers in the blood of *Periplaneta* (Mills, 1967; Mills and Nielsen, 1967), the blood volume reaches a peak on the third or fourth day of the cycle. At the same time the blood protein concentration is higher than at other periods during the cycle; thus correction for blood volume changes accentuates, rather than compensates, for the protein changes in the blood.

The abrupt decrease in blood proteins prior to and during ootheca formation, which was not observed by gravimetric methods (Pratt, 1967), may be attributed to one or several possible causes. Fat body protein synthesis on the fifth day of the cycle is at least 40% lower than on other days in the cycle, and thus the low blood protein may represent in part a hiatus of protein secretion (Pan *et al.*, 1969: Bell, 1969a). Another possible reason for the drop in blood protein may be the utilization of blood proteins by the colleterial glands for the production of the ootheca, a proteinaceous structure which weighs 20 mg. Electrophoretic protein fractions common to the blood and to the colleterial glands have been demonstrated in *Periplaneta* (Adiyodi and Nayar, 1966) and in *Nauphoeta* (Adiyodi, 1968). The structural proteins of the ootheca, however, do not have counterparts in the blood, and Adiyodi concludes, in agreement with earlier work by Pryor (1940), that the left colleterial gland sequesters and hydrolyzes specific blood proteins to obtain amino acids for the synthesis of structural ootheca proteins. In analogous work, Brunet (1952) has reported that the composition of the cuticle and ootheca of *Periplaneta* are biochemically similar, and at least one blood protein disappears from the blood during the molt in several insects (Steinhauer and Stephen, 1959; McCormick and Scott, 1966; Chen and Levenbook, 1966). Sequestration of specific blood proteins by the colleterial glands, as in other insect tissues (Locke and Collins, 1967; Laufer and Nakase, 1965), might explain the decrease in *Periplaneta* blood proteins during ootheca formation.

The author is grateful to Dr. William H. Telfer for his invaluable advice during the course of this study. This work was supported by research grants from NSF (GB-4463) and NIH (5-TO1-GM-00849-07).

SUMMARY

1. The 5-day reproductive cycle of *Periplaneta* is continuous with an ootheca being formed every fifth day. The rate of yolk formation is lowest during ootheca formation (day 1) and reaches a peak during days 3 and 4.

2. Vitellogenic blood protein levels fluctuate between 1 and 6 μg/μl and their concentrations correlate with the rate of yolk formation. The two vitellogenins oscillate synchronously during the cycle.

3. The total blood protein concentration increases to 80 μg/μl on day 5, decreases abruptly to 52 μg/μl when ootheca formation is initiated, and increases gradually to the concentration peak on day 5. The changes in total blood protein concentration are not obviously correlated with the vitellogenic cycle.

4. Changes in blood volume occur during the cycle, and these changes amplify rather than diminish the blood protein fluctuations.

5. A hiatus in food consumption is observed during ootheca formation; feeding is resumed after the third day following oviposition.

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THE PREZOEAL STAGE OF THE DUNGENESS CRAB, *CANCER MAGISTER* DANA[1]

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There is controversy in the literature as to the normalcy of the occurrence of a free prezoeal stage in the life history of the Dungeness crab, *Cancer magister*. MacKay (1942) stated that the eggs hatched into protozoeae (=prezoeae), but others reported that zoeae normally emerged from eggs and that free prezoeae were abnormal and died (Mir, 1961) or resulted from premature hatching of eggs (Poole, 1966). Our preliminary observations on egg hatching and on subsequent molting of prezoeae supported MacKay's belief. Before we could begin studies on the effects of the insecticide Sevin on survival and growth of *C. magister* larvae we had to resolve the controversy. This paper reports the results of a study undertaken to determine if salinity (1) affects egg hatching, (2) determines the type of larva that emerges from the egg, and (3) affects development of these larvae.

MATERIALS AND METHODS

Ovigerous crabs caught in the ocean off the Oregon coast were held in the laboratory in flowing sea water for 3 days. One crab was then transferred to a 31-gallon tank containing filtered, sterilized, standing sea water of 30‰ at 11° C and it was held for an additional 3 days. Eggs began to hatch at the end of this time. We could predict from previous experience that eggs would hatch within a few days when egg coloration changed from a light to a dark brown. When hatching began, about 2000 eggs of normal appearance with all cuticular layers and ovigerous setae intact were gently removed in small bunches from the crab and held for 3 hours in filtered, sterilized sea water of 32‰ salinity at 10.5° C. Only a few eggs hatched during this time and the larvae, some of which may have hatched prematurely because of the recent egg handling, were discarded. At the end of the 3 hours, unhatched eggs still attached to ovigerous setae and with their cuticular layers intact were then selected at random and separated into 14 groups of 20 and each group was placed into a 250 ml beaker containing sea water at 10.5° C and of one of the following salinities: 10, 15, 20, 25, 30, and 32‰. A temperature-salinity combination of 17.5° C and 32‰ was also tested. Duplicate vessels were used for each test. The larvae, upon hatching, were immediately transferred to another beaker containing sea water of the salinity and temperature at which they had hatched. All vessels were held under constant light. Eggs were

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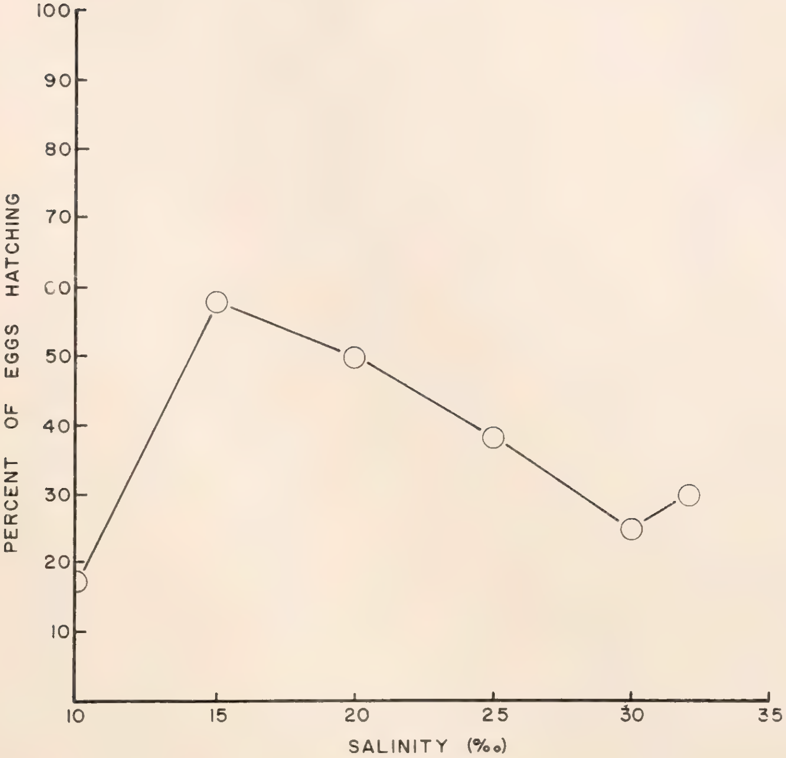

FIGURE 1. Effect of salinity on hatching of *Cancer magister* eggs at 10.5° C.

examined for hatching at intervals of about 5 minutes for 36 hours, and the hatched larvae were observed for molting at the same intervals for the first hour after they had hatched and then again at the end of the 36-hour experiment.

RESULTS

Some eggs hatched at all the test salinities, and 94% of these are known to have hatched into prezoeae (Table I). Of the remainder (6%), all of the eggs but one were in the two highest salinities at which the prezoeae may molt to zoeae as early as 2 minutes after hatching. Therefore, we believe that all of the hatching eggs hatched into prezoeae, but because of the short duration of this larval stage, especially at the higher salinities, some prezoeae could have hatched and molted between observations and thus they would not have been seen. The percentage of eggs hatching at 10.5° C increased as salinity decreased to an optimum of 15‰, but at 10‰ was lowest (Fig. 1). At a salinity of 32‰, the mean percentages of eggs that hatched at 10.5 and 17.5° C were 30 and 73%, respectively, indicating a marked temperature effect (Table I).

The mean percentages of prezoeae that molted to zoeae increased with increasing salinity from 0% at 10‰ to 100% at 30 and 32‰ (Table I and Fig. 2). With

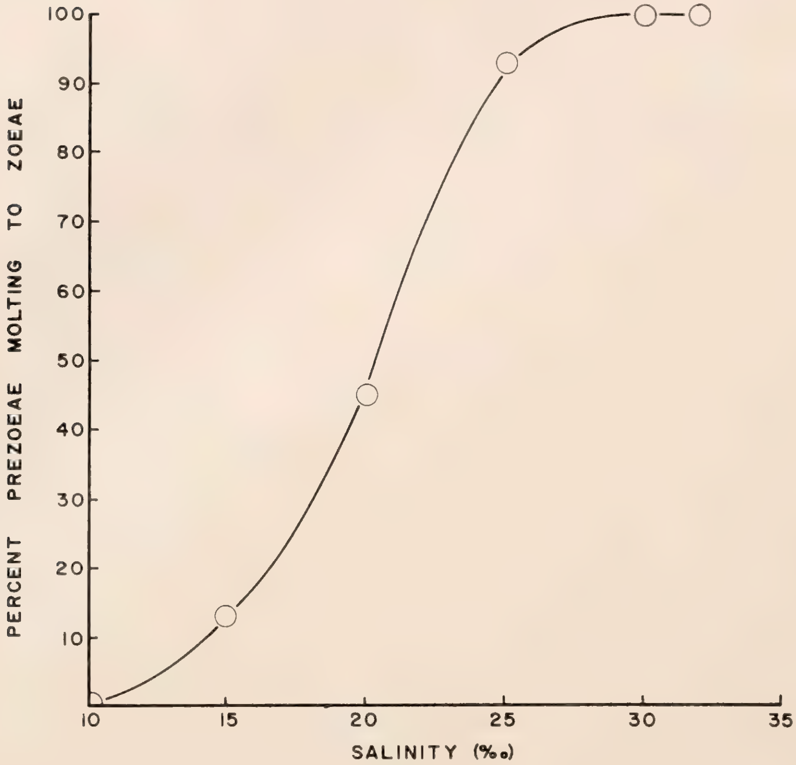

FIGURE 2. Effect of salinity on molting of *Cancer magister* prezoeae to zoeae.

increase of salinity from 15 to 32‰, the mean duration of the prezoeal stage decreased from more than 60 minutes to 11 minutes (Table I). At 32‰ salinity, all prezoeae molted to zoeae at the two test temperatures, and there was no effect of temperature on duration of the prezoeal stage.

Our morphological observations of the prezoeae agree with those of MacKay (1934) except that we did not see the lateral spines on the cephalo-thorax. The prezoeae have large eyes, a five-segmented abdomen, and a forked telson with spines (Fig. 3). They lack the rostral and dorsal spines of the zoeae. A further distinguishing characteristic, not mentioned by MacKay (1934), is the shortness of the natatory hairs on the maxillipeds. These hairs on the maxillipeds of the zoeae are much longer.

Prezoeae swam erratically, using their abdomen and telson for propulsion. As first noted by MacKay (1942) their movement resembled that of a mosquito larva. It was weak at the intermediate salinities and at 10 and 15‰ salinity the prezoeae did not swim.

Molting of prezoeae to zoeae required only a few seconds. The prezoeae first settled to the bottom of the container; then they extended the maxillipeds, the cuticle split, and the dorsal and rostral spines emerged. Our first-stage zoeae conformed morphologically with those described by Mir (1961) and Poole (1966).

TABLE I

Effects of salinity and temperature on hatching of Cancer magister *eggs and on molting of the prezoeae to zoeae**

Salinity (‰)	Hatched eggs		Free prezoeal stage confirmed		Duration of prezoeal stage (min)**		Prezoeae molted to zoeae	
	No.	%	No.	%	Mean	Range	No.	%
10	4	20	4	100	—	—	0	0
10	3	15	3	100	—	—	0	0
15	12	60	12	100	>60	—	1	8
15	11	55	11	100	>60	—	2	18
20	9	45	8	89	>44	13–>60	3	36
20	11	55	11	100	>25	9–>60	6	54
25	8	40	8	100	14	7–31	8	100
25	7	35	7	100	14	7–27	6	86
30	6	30	4	67	10	7–14	4	100
30	4	20	3	75	4	2–7	3	100
32	4	20	4	100	12	9–18	4	100
32	8	40	7	88	10	6–16	7	100
32†	17	85	15	88	11	3–35	15	100
32†	12	60	12	100	11	4–30	12	100

* Twenty eggs were used in each test and they were observed for hatching approximately every 5 minutes for 36 hours. All tests were done at 10.5° C unless noted otherwise.

** Hatched prezoeae were observed for molting approximately every 5 minutes for the first hour after they had hatched and again at the end of the 36-hour experiment.

† Test temperature was 17.5° C.

Five zoeae, which had developed from prezoeae, were randomly selected at the end of the experiment and held in sea water of 32‰ at 10° C for 3 days. All of them survived and appeared normal during this time.

DISCUSSION

Our observations support the contention of MacKay (1934) that the free prezoea is a normal stage in the life history of *C. magister* and not the abnormality that it was believed to be by Mir (1961) and Poole (1966). The results of our study support the statement of MacKay (1942) that the prezoeal stage is of short duration. This short duration may explain Poole's (1966) failure to see prezoeae, his observations having been made apparently no more frequently than once a day. Mir (1961), who also apparently observed his eggs no more frequently than once daily, stated that prezoeae, when present, were "imperfect" and died. He saw prezoeae apparently only when eggs were refrigrated or contaminated with protozoa and undoubtedly for these reasons his prezoeae were indeed abnormal and died without molting.

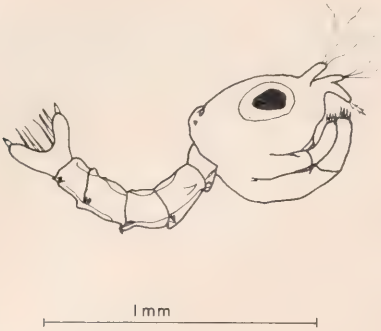

FIGURE 3. Prezoea of *Cancer magister* after hatching from the egg.

Churchill (1942), on the basis of laboratory studies, stated that a free prezoeal stage, which lasted from 30 to 60 minutes, was normal in the life history of the blue crab, *Callinectes sapidus*. However, Sandoz and Rogers (1944) believed that their experimental data did not support Churchill's statement. They concluded that unfavorable environmental conditions, such as suboptimal salinities, were responsible for the occurrence of free prezoeae, because in their experiments the numbers of free prezoeae increased as salinities decreased. They found no prezoeae when eggs were hatched at salinities ranging from 23.4 to 32‰, but at a salinity of 10‰, 90 to 100% of the hatched larvae remained as prezoeae. We found the same for *C. magister* prezoeae at low salinities, but at high salinities all of our prezoeae molted to zoeae. Sandoz and Rogers (1944) observed their eggs only twice daily, and therefore they could have failed to see the short-lived prezoeae at the higher salinities. The data of Sandoz and Rogers (1944), therefore, do not refute the belief of Churchill (1942), and for the same reason neither do the data of Costlow and Bookhout (1959), who also reported that eggs of *C. sapidus* at salinities ranging from 20.1 to 32‰ always hatched as first-stage zoeae. Costlow and Bookhout (1960) observed the eggs of six species of Brachyura, including *C. sapidus*, once daily and reported that most of the eggs of all species hatched as zoeae. Knudsen (1959) and Williams (1968) found that eggs of the crabs *Paraxanthias taylori* and *Carcinus maenas*, respectively, normally hatched as prezoeae, and the latter author reported that the average duration of the prezoeal stage of *C. maenas* was 4 to 5 minutes. It is clear, therefore, that the early life history of the blue crab and of other crabs in which the normal occurrence of a free prezoeal stage is doubted must be restudied.

The range of salinity that proved optimal for molting of our prezoeae was between 25.0 and 32‰ at 10.5° C; at 32‰ salinity, prezoeae molted equally well at 10.5 and 17.5° C. Reed (1969) found that the optimal salinities and temperatures for development in the laboratory of *C. magister* first-stage zoeae to the megalops stage were between 25 and 30‰ and between 10.0 and 13.9° C.

We wish to thank Mr. Paul H. Reed, Fish Commission of Oregon, for suggesting to us the possibility that free prezoeae are normal in the life history of *C. magister*, and Mr. Dennis E. Anderson and Mr. Nelson E. Stewart, Oregon State University, for making some of the observations.

SUMMARY

1. Ninety-four per cent of eggs of the Dungeness crab, *Cancer magister,* held in sea water at 10.5 or 17.5° C and at salinities of 10 to 32‰ hatched into prezoeae during a 36-hour observation period. The highest and lowest numbers of eggs hatched at salinities of 15 and 10‰, respectively. At a salinity of 32‰, the mean percentages of eggs that hatched at 10.5 and 17.5° C were 30 and 73%, respectively.

2. With increase of salinity, the percentages of prezoeae that molted to first-stage zoeae increased from 0% at 10‰ to 100% at 30 and 32‰.

3. With increase of salinity from 15 to 32‰, the mean duration of the prezoeal stage decreased from more than 60 minutes to 11 minutes.

4. The experimental results show that the occurrence of a free prezoeal stage of short duration is normal in the life history of *C. magister.* The possibility that this is true for other Brachyura is discussed.

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PHOTOPERIODICALLY INDUCED TESTICULAR RECRUDESCENCE IN THE TRANSEQUATORIAL MIGRANT *DOLICHONYX* RELATIVE TO NATURAL PHOTOPERIODS [1]

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Some annual cycles of many birds of the northern hemisphere are known to be controlled by seasonal changes in the length of the daily period of light, that is, by the natural photoperiod. Best known is the annual cycle of the male gonad (reviews by Burger, 1949; Farner, 1959, 1961). Transequatorial migrants present a special problem, since they twice annually pass from the shortening days of autumn in one hemisphere to the lengthening days of spring in the other. They never experience the short days of late autumn and early winter; every year they experience two spring-summer seasons, but they confine their nesting to just one of these. Obviously, if the annual day-length cycle plays a part in regulating their annual breeding cycle, then the mechanism must differ in some conspicuous way from that operative in a temperate-zone resident or migrant. Early speculations as to the nature of this difference (*e.g.,* Farner, 1954) focused on the post-breeding photorefractory phase of the cycle and assumed that the migrants would still be refractory on arrival in the other hemisphere, where then lengthening days might prolong refractoriness until the late summer.

A photoperiodic effect on a transequatorial migrant was first demonstrated (Engels, 1959) in the bobolink, *Dolichonyx oryzivorus,* which breeds in North America in June and July and spends the months of November through March in South America below the equator. The testicular cycle in this species shows a photorefractory phase which in some respects is like that of temperate-zone migrants, while in others it differs in a manner that suggests it would be terminated during the post-breeding, southward transequatorial migration (Engels, 1961, 1962). There is a suggestion that, following termination of refractoriness, initiation of recrudescence is greatly delayed in comparison with temperate-zone migrants on moderately long photoperiods, but apparently begins abruptly, without delay, on quite long photoperiods, as in temperate-zone migrants (Engels, 1964). It is the purpose of the present paper further to examine these last two points, and to attempt a general correlation of the experimental results with the events occurring in nature.

Accordingly, this report is divided into two main sections, the first of which describes the experiments and their results, and makes some comparisons with a temperate-zone migrant; following this is an account of the distribution of bobolinks in South America, the time of occurrence of testicular recrudescence, and the relationship of the experimental results to the timing of this event in terms of day-lengths encountered in nature.

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MATERIALS AND METHODS

Ninety-six male bobolinks were used in these experiments. Of these, sixty-two were caught in May during northward migration, hence all were about one year old or older. Thirty-four birds were caught in September during southward migration; many of these were adults but some were immature. All the autumn captures and nine of the spring captures were taken in North Carolina; fifty-three birds were caught in Florida in early May and transported to Chapel Hill by commercial airline. All the birds were held in an outdoor aviary until the start of artificial lighting in early October, and hence were exposed until then to the natural photoperiods of Latitude 36° N.

At the beginning of October the birds were confined in cages in ventilated, light-tight compartments in the attic of the laboratory. "White" fluorescent lamps, controlled by automatic time switches, provided from 95 to 135 foot candles of light on the floor of each cage, depending on its position in the compartment. It is presumed that these intensities are supramaximal for the response studied. Temperature was not controlled; in the unheated attic, within the lighted compartments, the temperature ranged from 18° to 25° C, approximately, during the course of the experiments. There was a day-night difference of about 2° C due to heat from the lamps. A food hopper and two water tubes provided ample food and water. The food consisted of a commercial, pelleted mash prepared for "game" birds.

During the first five weeks of artificial lighting, all birds were exposed to daily photoperiods of 12.5 hours (0545h–1815h). Previous study had shown that photorefractoriness would be terminated under such a lighting regime (Engels, 1962). Four of the September captures and six of the May captures were then killed; the weights of their testes indicated the approximate weights of testes of the remaining birds at the start of exposure to longer photoperiods.

In all my earlier studies on bobolinks, a "long" day of 14 hours of light was used and the occurrence of recrudescence was scored for each bird when it became apparent that the horny epidermis of the beak was darkening. This black pigmentation, characteristic of the male in nuptial dress, is caused by the male sex hormone (Engels, 1959). We did not know, of course, what was happening previous to the appearance of pigmentation. In the present experiment the remaining thirty autumn-caught birds were exposed to 14-hour daily photoperiods and, beginning after six weeks, were sampled at 14-day intervals. Four of the spring-caught birds were killed after only sixteen days on 14-hour photoperiods. The remaining 52 spring-caught birds were divided into 4 groups which were exposed to longer photoperiods, namely 18 hours (six birds), 17 hours (twelve birds), 16 hours (seventeen birds) and 15 hours (seventeen birds). These were sampled first after 16 days, when all the "18-hour" birds had developed beak pigmentation; further sampling was done on a plan intended to uncover differences between photoperiods in the rate of recrudescence.

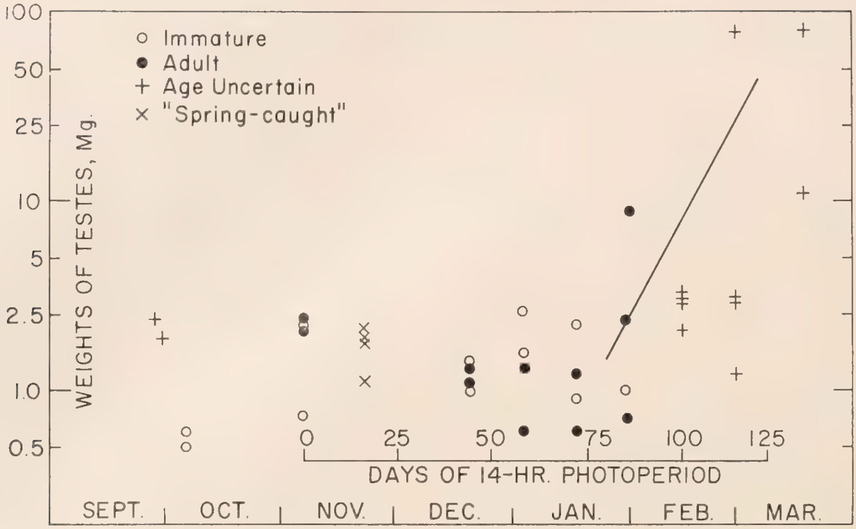

FIGURE 1. Recrudescence of bobolink testes, as indicated by weight, when exposed to 14-hour photoperiods beginning in early November. Sloping line indicates a *k* value of 0.04 beginning at day 80.

The birds were killed by decapitation, the testes removed and fixed in Helley's fluid; subsequently, after some time in 70% alcohol, they were weighed to the nearest 0.1 mg.

RESULTS

The results are presented in Figures 1 and 2. It will be convenient to consider them in three steps: first, the data from the 14-hour photoperiods, next the data from the 18-hour photoperiods and lastly the data from the photoperiods intermediate to these two.

1. *14-hour photoperiods* (*Figure 1*)

There is no apparent upward trend in the testicular weights until 84 days after the first 14-hour photoperiod, when one of the four birds shows that recrudescence has begun, with testes weighing 8.9 mg. After 114 days the testes of one of four birds are still inactive (only 1.2 mg), two may have just begun active growth (about 3 mg), while one has reached 80 mg. The final two specimens, killed after 130 days, were both undergoing testicular recrudescence, although the testes of one weighed only 11.1 mg. The results indicate that the initiation of recrudescence is delayed for several weeks, perhaps ten or more, after the first 14-hour photoperiod.

2. *18-hour photoperiods* (*Figure 2*)

The results are in sharp contrast to those of the 14-hour group. Recrudescence apparently began immediately; after only 16 days each of six birds tested had

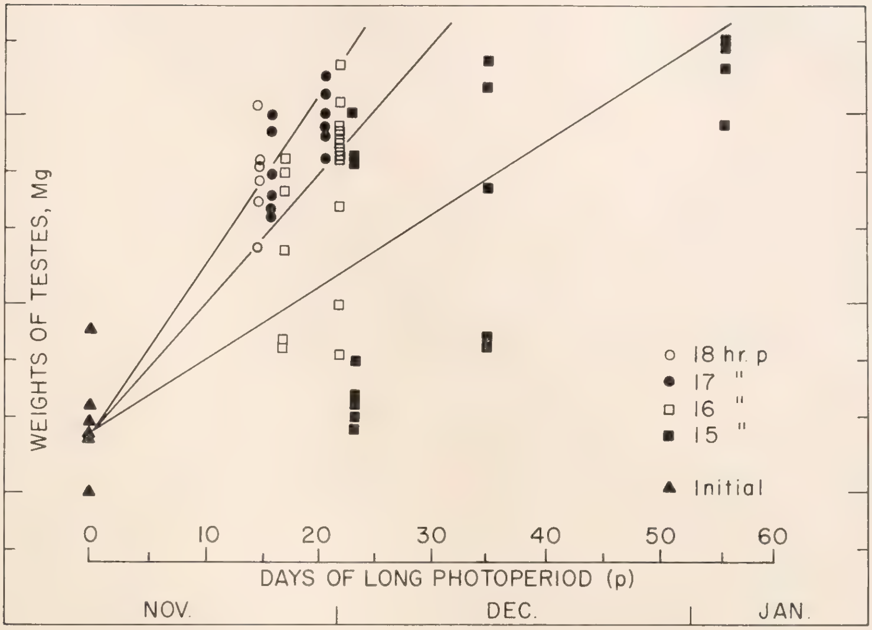

FIGURE 2. Recrudescence of bobolink testes, as indicated by weight, when exposed in early November to photoperiods of 15, 16, 17 and 18 hours. Lines indicate *k* values of 0.09, 0.07 and 0.04 (see text).

already developed the black beak pigmentation indicative of the production of male sex hormone, and testicular weights ranged from about 20 mg to more than 100 mg. Four birds of similar history, which were killed at the same time after 16 days on 14-hour photoperiods, each had testes weighing less than 2.5 mg (average 1.75 mg, Figure 1).

3. *17-hour, 16-hour and 15-hour photoperiods* (*Figure 2*)

Although the "17-hour" birds lagged a little behind the "18-hour" birds with respect to average time of development of beak pigmentation, the samples do not show any marked difference in the rate of recrudescence. On the 16th day, weights of testes of six birds in the "17-hour" group ranged from about 30 mg to just over 100 mg (compared with 20 mg to 115 mg in the "18-hour" group). At this time there still were six birds in the "17-hour" group that did not show beak pigmentation; by the 22nd day their testicular weights considerably exceeded in value those of the first sample, ranging from 60 mg to 164 mg, with an increase in the mean from 56 mg to 104 mg.

The rate of recrudescence in the "16-hour" group was definitely lower than in the previous two groups, weights of six pairs of testes on the 16th day ranging from 6 mg to 60 mg. By the 22nd day some were still quite small (5.5 mg and 10 mg in two birds) but a number were of the same size as in "17-hour" birds at this time (17-hour: 60 mg to 164 mg; 16-hour: 59 mg to 189 mg). The first sample of the "15-hour" birds was taken on this day; the range of testicular weights (2.3 mg to 105 mg) is somewhat lower than in the "16-hour" group, and the median is much lower (5 mg compared with 67 mg), both indicating a lower rate of recrudescence. After 35 days testes of two birds still were less than 10 mg, but testes of the other three birds weighed from 90 mg to just under 250 mg.

Initial weights (*Figure* 2)

The testes of five (spring-caught) birds had an average weight of 2.0 mg (1.0 mg to 2.8 mg) after the five weeks of 12.5-hour photoperiods in the early fall. In a sixth one the testes astonishingly were enlarged and weighed 7.2 mg. At this time I cannot account for this exceptional individual. Nevertheless the general conclusions presented above and the analysis to follow, which exclude any consideration of this one bird, seem to me to be truly valid.

Comparison with a temperate-zone migrant (*Figures 1 and 2*)

The sloping lines in Figures 1 and 2 are not fitted to the plotted data; rather, they are drawn to three selected values for average daily increment in the logarithm of weights in milligrams (k). These selected values approximate those determined for *Zonotrichia leucophrys,* a temperate-zone migrant, for photoperiods of 18 hours (0.09), 16 hours (0.07) and 14 hours (0.04) (Farner and Wilson, 1957). They are intended only to serve as visual guidelines, to aid in evaluating the presented bobolink data and in making general, qualitative comparisons between these two species.

The data both from 18-hour and from 17-hour photoperiods indicate an immediate, linear response of the same general order of magnitude as in *Z. leucophrys* for these photoperiods. The same probably may be said of the data for 16-hour photoperiods. At least some of the birds on 15-hour photoperiods also indicate an immediate response, with recrudescence proceeding then at a somewhat low rate. On 14-hour photoperiods, however, quite unlike temperate-zone migrants, the testicular response definitely is delayed for several weeks, ten or more; but once recrudesecence is initiated, it proceeds at a rate which may perhaps be comparable to the rate of the immediate response of *Z. leucophrys* to this photoperiod (Fig. 1). The delay is rather to be expected in a transequatorial migrant; after all, there is an interval of about seven months (in this particular case) between arrival in the southern hemisphere in early November and the next courtship, mating and nesting in the following June in the northern hemisphere.

DISCUSSION

Annan (1963) not only still entertained the old view that this delay was the expression of a photorefractory condition, but cited results (Engels, 1962) with bobolinks as "supporting" the idea. The data now at hand for the longer photoperiods, which induce an immediate response, show that refractoriness had indeed been terminated by the preliminary treatment of five weeks of daily 12.5-hour photoperiods. Whatever the mechanism responsible for the delayed response to 14-hour photoperiods, it cannot be said to be photorefractoriness. Thus, the mechanism of recrudescence in this transequatorial migrant differs from that in a temperate-zone migrant *not* in a prolongation of the photorefractory period, but first in the capacity to terminate refractoriness on much longer days (such as are encountered in the transequatorial passage), and secondly in the delayed response to moderately long photoperiods.

1. *Distribution from November through March in South America*

Meyer de Schauensee (1966, p. 441) states that the bobolink is a "winter resident in southern South America in Peru, eastern Bolivia, western Brazil (Amazonas; Mato Grosso; casually in Rio Grande do Sul), Paraquay and nothern Argentina to Tucumán, Córdoba, Santa Fe and Buenos Aires." The latitudinal limits thus lie in Peru and Argentina. In Peru bobolinks occur from Tujillo, Latitude 8° S., southward (Koepke, 1961). The Argentine limits as outlined above repeat the distributional data given by the Argentine ornithologist Olrog (1963). In reply to my inquiry, Olrog writes that "the normal southern limit goes over southern Tucumán, northern Córdoba, Santa Fe and Entre Rios." This would put them down about to Latitude 30°–32° S. He adds that "stragglers go farther south, eventually attracted by crops" but he believes that the "normal winter home is well in the subtropical lowlands and not in the temperate." The inclusion of the province of Buenos Aires in both lists (Meyer de Schauensee, 1966; Olrog, 1963) probably is based on a published record by Pereyra (1923); he lists a male collected in December, 1920, at Isla de Escobar, Province of Buenos Aires (about 34° S.?) and remarks that "en ciertas epocas" (Pereyra, 1923; p. 171) the species appears in this region in large flocks. In his letter to me Olrog says, "personally, I never saw a bobolink in the Buenos Aires province."

It appears, then, that during their sojourn in South America bobolinks occur at localities lying between Latitude 8° S. and Latitude 30° to 32° S., with occasional or sporadic wandering of some flocks farther southward, perhaps as far as to Latitude 35° S.

2. *Testicular recrudescence in nature in South America*

The evidence as to when testicular recrudescence occurs in nature is inferential and is based on museum specimens. From August to March male bobolinks are grossly indistinguishable from females. During the breeding season, however, the male is a strikingly colored bird of black, white and golden cream with a jet-black beak, totally unlike the streaked, pale brown and yellowish female with a pale, horn-colored beak. The molt to the nuptial plumage occurs in South America, beginning in late January or in February; it is completed sometime in March (exceptionally in late January), before the start of the prenuptial, northward migration. There is no evidence that the coloration of the new plumage is linked in any causal way with active testes. On the other hand, the nuptial beak pigmentation is so linked; it is caused directly by the male sex hormone (Engels, 1959). Hence, the appearance of this pigmentation is a sign that testicular recrudescence already is under way.

Of the November-March specimens in U. S. museums, none of the males has a darkened beak, although four are in full breeding plumage (31 January, 23 and 28 March and 1 April; all collected between 17° and 22° S.). There are twelve additional April males (all migrants) from South America, all in full breeding plumage. Of these, four have darkly pigmented beaks, eight do not. Those with

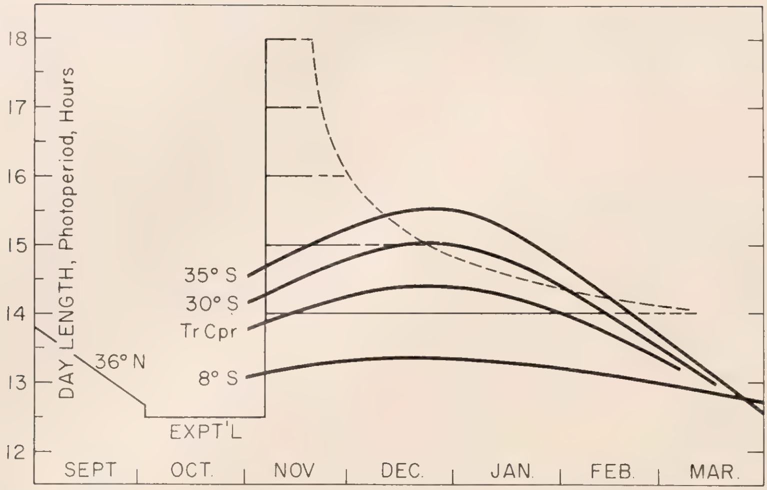

FIGURE 3. Day-lengths (including civil twilight) experienced by bobolinks in South America (curved lines) and the photoperiods used in these experiments (straight lines). The broken-line curve approximates the occurrence of testicular regrowth to weights of about 50–100 mg, Tr Cpr—Tropic of Capricorn.

dark beaks were collected above the equator in Colombia, April 14–22, 1957; of those with pale beaks, five were taken on April 16, 1923, in Brazil about 4° below the equator, one on April 26, 1903 above the equator in Venezuela while one from Brazil and one from Venezuela lack precise dates. Since, then, mid-April may approximate the first appearance of this pigmentation, the testes must remain inactive perhaps through March, with the initiation of recrudescence occurring at about the beginning of April.

3. *Day-lengths in South America and the photoperiodic response of male bobolinks (Figure 3)*

If the photoperiodic responses obtained in our experiments are to be meaningful, it is necessary to relate them to the natural photoperiods experienced by the species. Unfortunately, the results are not entirely unequivocal.

The most satisfactory "fit" of the experimental data to winter distribution has to do with the southern limits. Since the winter range does not extend beyond Latitude 30° to 35° S., it is clear from Figure 3 that the males are never exposed, even at the December solstice, to days as long as or longer than 16 hours—the photoperiods which in our experiments induced almost immediate recrudescence. In effect, the response to these longer photoperiods would act selectively against a southward extension of the species' range into the higher latitudes characterized by these longer day-lengths.

On the other hand, the results of the experiment using 15-hour photoperiods would suggest that the winter range should not extend to Latitude 35° S. Indeed, in an earlier experiment (Engels, 1962; Group E, p. 97), I did use photoperiods which simulated the daily change in day-length at Latitude 35° S.; testicular recrudescence was induced apparently by late December (beak pigmentation obvious by mid-January), much too early compared with the natural timing. Fourteen-hour photoperiods approximate average natural day-lengths in the northerly part of the winter range, and they did greatly delay the onset of recrudescence, although not quite until the normal time.

In this connection, there is an interesting question (but one not yet investigated, as far as I know): whether, in the various species which show photoperiodic responses, there exist intraspecific differences in threshold or in rate of response between populations wintering at different latitudes. It is not unreasonable to think that such differences may exist. Of course we do not know where, in South America, our experimental birds had their winter homes. If they came from relatively low latitudes (north of the Tropic of Capricorn for example), and if there are differences in rates of recrudescence correlated with latitude, then the experimental results might approximate more closely those expected from a consideration of events in nature.

For information regarding details of distribution in Argentina I am indebted to J. G. Esteban and C. C. Olrog, Instituto Miguel Lillo, Tucumán, and to J. R. Navas, Museo Argentino de Ciencias Naturales, Buenos Aires.

Summary

1. *Dolichonyx oryzivorus,* the bobolink, breeds in North America in June and July and spends the months of November through March in South America below the equator. Ninety-six males captured during migration were held in an outdoor aviary at Chapel Hill, North Carolina (Latitude 36° N) until start of artificial lighting.

2. Beginning in early October, they were exposed daily to 12.5-hour photoperiods for five weeks to terminate photorefractoriness. Thereafter, different groups were subjected to daily photoperiods of 14, 15, 16, 17 and 18 hours. These groups were sampled at intervals and progress of testicular recrudescence was determined by gonadal weights.

3. Evidence adduced from museum specimens indicates that testicular recrudesence begins about the end of March. In the experiments, recrudescence seemingly was initiated immediately, in November, on the longer photoperiods and proceeded at rates comparable to those shown by a temperate-zone migrant. Drastically different was the response to 14-hour photoperiods, initiation of recrudescence being delayed for ten or more weeks.

4. In South America bobolinks occur between Latitude 8° S and Latitude 30° to 35° S. Day-lengths at these latitudes never exceed 15.5 hours; so even at the December solstice males are not exposed to the longer photoperiods which brought about immediate recrudescence in the experiments. Because bobolinks do not come into breeding condition in the southern hemisphere, this response is viewed as a limiting factor in southward distribution. But since 15-hour photoperiods also induced early recrudescence, one would not expect the species to go as far south as Latitude 30° to 35°. This is the most puzzling lack of fit of the experimental results to nature.

5. Although 14-hour photoperiods greatly delay the onset of recrudescence, they do induce it earlier than its occurrence in nature, even though they are some-

what shorter than the average day-length occurring at the latitudes in the middle of the bobolink's winter range. Thus there is at best an imperfect fit of the experimental results to the normal timing.

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STUDIES ON A TREHALASE FROM A SYMBIONT OF THE TROPICAL COCKROACH, *BLABERUS CRANIIFER*—A SUGGESTED ANALYTICAL ENZYME[1]

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Herein we report the characterization of a trehalase (α,α-1-glucoside 1-glucohydrolase, EC 3.2.1.28) isolated from a bacterial symbiont from the midgut of *Blaberus craniifer,* a tropical cockroach.

Although this is not the first isolation of such a hydrolase, the present study will disclose that this source of enzyme has one advantage over previously reported sources, *i.e.,* crude preparations of this enzyme do not contain large amounts of any other carbohydrases which would release glucose from glucose-containing oligosaccharides or polysaccharides and thus interfere with the qualitative or quantitative estimation of trehalose in complex biological fluids or extracts. The absence of other hydrolases simplifies purification procedures and insures the accurate quantitative estimation of trehalose in mixtures containing such oligosaccharides and polysaccharides.

Materials and Methods

Culture conditions

The microorganism, a gram negative bacillus, apparently a species of *Aerobacter,* was obtained from the midgut of *Blaberus* and isolated by routine plating techniques on minimal salt media where trehalose served as the only carbon source. The isolated strain was grown in minimal salt medium (Heath and Ghalambos, 1962) plus 5% trehalose at 34° in a rotary incubator for 24–36 hours. The cells were removed by centrifugation at 46,000 *g* for 30 minutes at 2° and washed by further centrifugation in the minimal salt medium without carbohydrate.

Isolation and purification of the trehalase

The washed cells were take up in distilled water (1:3 w/v) and stored at −20° overnight. The cell suspension was thawed and refrozen three times and subjected to intermittant sonication for six minutes at 0°. Cell debris was removed by centrifugation at 50,000 *g* at 0° for 20 minutes and the clear supernatant adjusted to 0.05 *M* with $MnCl_2$. This preparation was maintained at 0° for 30 minutes and cleared by centrifugation at 30,000 *g* at −1°. The resultant supernatant was brought to 30% (v/v) with −20° reagent grade acetone and the mixture held at −7° for five minutes before centrifugation at 30,000 *g* at −10°. The acetone supernatant was discarded and the precipitate suspended in 50 ml 0.001 *M* citrate

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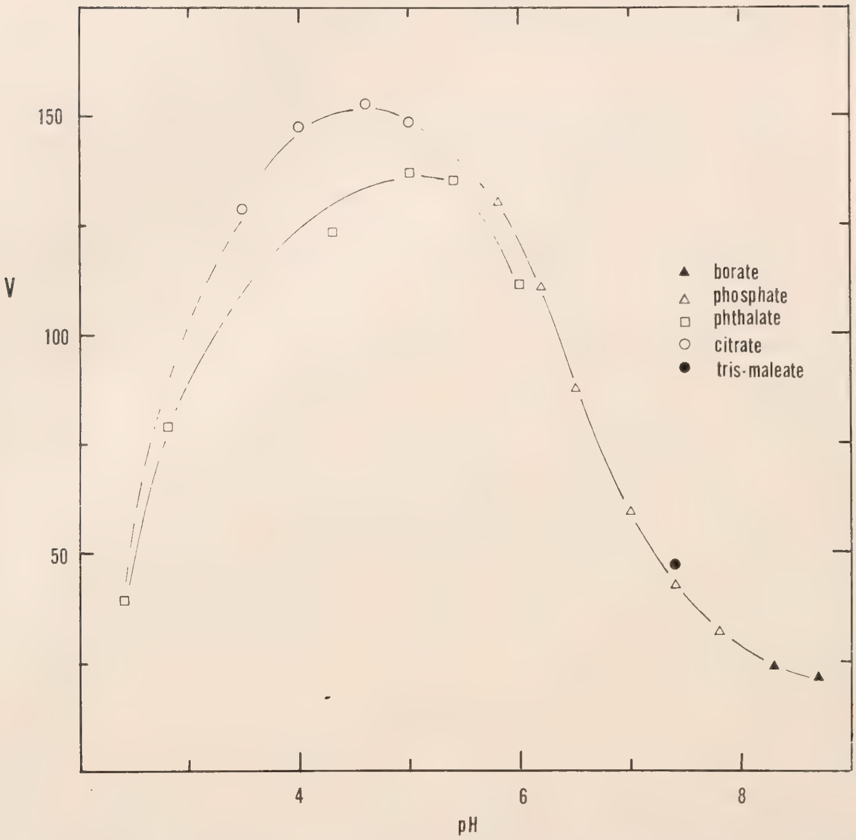

FIGURE 1. Effect of pH on trehalase activity; incubation mixtures contained in a final volume of 0.325 ml: 10 μmoles trehalose, 50 μmoles buffer and 0.025 ml enzyme.

buffer at pH 5.0. After gentle stirring at 0° for 30 minutes the undissolved material was removed by centrifugation at 46,000 × g at 0° for 30 minutes. The acetone fraction was then subjected to column chromatography at 3° on DEAE cellulose prepared according to the method of Peterson and Sober (1956). The 10 ml column was equilibrated with 0.005 M phosphate buffer pH 7.4 and stepwise elution of the enzyme was effected by the addition of 30 mls of each the following buffers at 0.005 M: phosphate pH 7.4 and 6.5 followed by citrate at pH 5.8 and 4.6. Following elution with the above buffers, a second elution of 0.005 M citrate buffer at pH 4.6 containing increasing amounts of NaCl was conducted in the following sequence: 0.01 M, 0.1 M, 0.5 M and 1.0 M. The eluted enzyme was dialyzed at 4° C against 0.005 M citrate buffer at pH 4.6 and the dialysate reduced to a powder by lypholyzation.

Assay of enzyme and analytical methods

The standard assay system contained 50 μmoles of buffer, 10 μmoles of substrate and 0.025 ml of enzyme in a total volume of 0.325 ml. Deviations from this

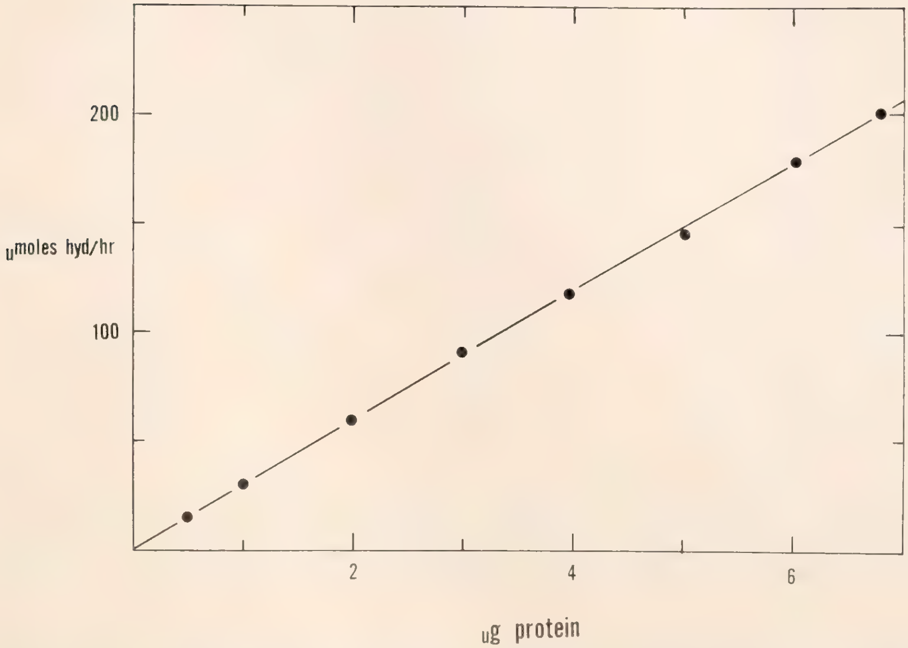

FIGURE 2. Effect of enzyme concentration on rate of trehalose hydrolysis; incubation mixtures contained in a final volume of 0.325 ml: 10 μmoles substrate, 50 μmoles citrate buffer at pH 4.6 and 0.025 ml enzyme.

assay system will be discussed in the text. Reaction mixtures were incubated for 15 minutes at 30°. To estimate the products formed in the assay system two techniques were employed: (1) Glucose-oxidase (Sols and de la Fuente, 1957) (β-D-Glucose: oxygen oxidoreductase, EC 1.1.3.4): o-dianisidine ("Glucostat," Worthington Biochemical Corp.) made up in 0.2 *M* Tris: HCl buffer, pH 7.2 (Fisher, 1964) was added to the reaction mixture, substrate blanks and glucose standards and incubated for 10 minutes at 30°. This reaction was stopped and the color developed by the addition of 0.05 ml of 8.0 *N* HCl. The tubes were read in a Coleman Universal spectrophotometer at 410 mμ or in a Klett-Summerson colorimeter with a #42 filter. (2) Alternatively the incubation mixture was subjected to the alkaline copper method of Somogyi (1952) for detecting reducing sugars. In addition some estimations were made exposing the reaction mixture to hydrolysis in 0.2 *N* NaOH at 100° for 20 minutes which degraded the enzymatically liberated glucose and the remaining trehalose was estimated by the phenol-H_2SO_4 method of Dubois, Gilles, Hamilton, Rebers and Smith (1956). In cases where protein interfered with the above estimations, the reaction was subjected to equal volumes of $Ba(OH)_2$ and $ZnSO_4$ (Somogyi, 1952). After clarification by centrifugation aliquotes of the supernatant were assayed by the glucose oxidase and/or the alkaline-copper method. Protein was estimated by the Folin-Ciocalteu reagent (Lowry, Rosenbrough, Farr and Randall, 1951) using crystalline bovine serum albumin as the standard. Enzymatic activity is expressed as μmoles substrate hydrolyzed/mg protein hr^{-1}.

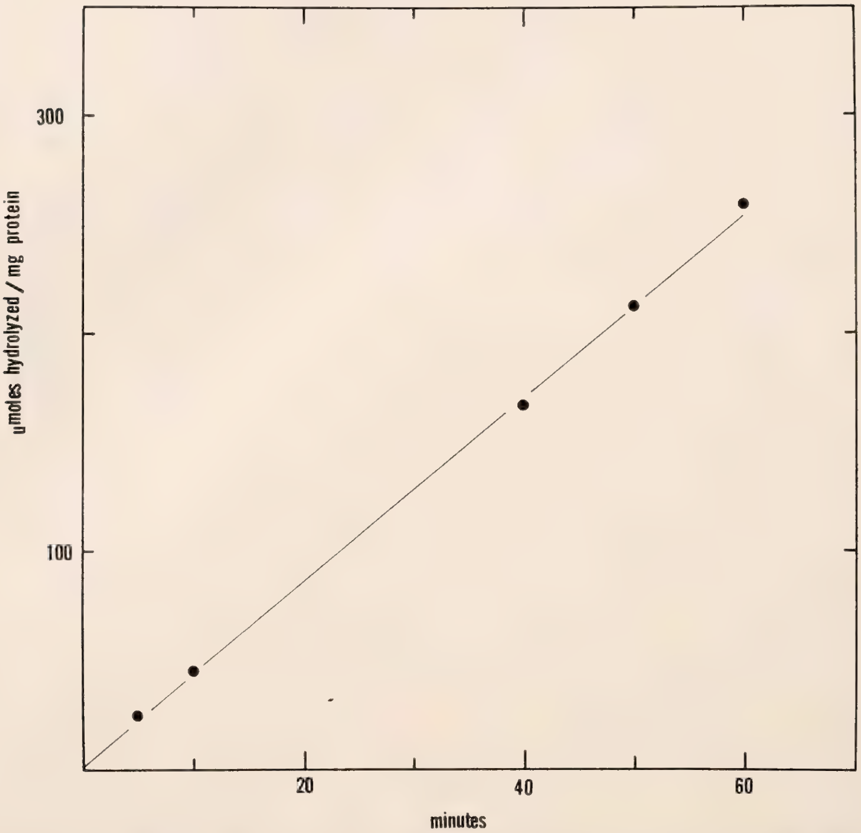

FIGURE 3. Effect of time on rate of trehalose hydrolysis; incubation mixtures contained in a final volume of 0.325 ml: 20 µmoles trehalose, 50 µmoles citrate buffer at pH 4.6 and 0.01 ml enzyme.

Chemicals

All chemicals used in these experiments were reagent grade. Substrates were purchased from Pfanstiehl Chemical Company and/or Sigma Chemical Company.

RESULTS

Effect of pH

Figure 1 illustrates the effect of pH on the rate of hydrolysis of trehalose. Data presented in that figure is based on the average of four determinations at each pH. The optimal pH in citrate buffer was found to be about 4.6. Phthalate buffers at the same concentration and covering the same pH range caused about 13% inhibition of enzymatic hydrolysis. No inhibition of hydrolysis was observed when Tris (hydroxymethyl) amino methane: maleate buffers were examined.

TABLE I

*Effect of metals on enzymatic activity**

Addition	Sp Act	%Inh
—	149.6	0
$CaCl_2$	136.8	8.6
$NiCl_2$	134.4	10.2
$CdCl_2$	119.2	20.4
$ZnCl_2$	110.4	26.2
$CoCl_2$	94.4	36.9
—	149.0	0
CsCl	100.0	32.9
LiCl	99.2	33.5
NaCl	96.0	35.6
KCl	96.0	35.6
RbCl	94.4	36.4
PCMB	147.0	0

* Incubation mixtures contained in a final volume of 0.525 ml: 50 μmoles citrate buffer, pH 4.6, 10 μmoles substrate and 0.025 ml enzyme. Divalent inhibitors were used at 10 μmoles, monovalent metals at 200 μmoles and PCMB at 0.033 μmoles. Mixtures were incubated for 15 minutes at 30° and the liberated glucose assayed by glucose-oxidase with inhibitor controls.

Effect of time, characterization of the product, effect of enzyme concentration and substrate concentration

Figure 2 indicates that enzymatic hydrolysis of trehalose was linear with respect to enzyme concentration over the range examined 0.05 to 7 μgm) and is based on triplicate observations. The product of hydrolysis was identified as glucose in four chromatographic solvents: butan-1-ol, pyridine, water (6:4:3); propan-1-ol, ethyl acetate, water (7:1:2); butan-1-ol, ethanol acetone, water (5:4:3:2); ethyl acetate, pyridine, 2-butanone, water (50:36:36:30). Following development of the chromatograms with alkaline silver nitrate (Trevelyan, Procter and Harrison 1950), only two spots were visualized, corresponding to glucose and trehalose. Eluted glucose areas were further examined by the alkaline copper reagent for total reducing sugar, glucose oxidase specific for glucose and total carbohydrate by the phenol:H_2SO_4 method. Results from these reactions gave the following ratios: 1.04:1.0:1.02.

Enzymatic hydrolysis was also linear with respect to time (Fig. 3) when examined over a 60 minute period. The rate of hydrolysis of trehalose was examined over a 100 fold range using triplicate tubes at each concentration (Fig. 4) and the K_m was estimated to be 2.24×10^{-3} *M*. The V_{max} was calculated to be 289.9 μmoles hydrolyzed/mg protein hr^{-1} from the double reciprocal plot (Figure 4) derived from the average of four determinations. Maltose, cellobiose, α-methyl glucoside and glucose at 0.05 *M* and starch and glycogen at 0.1 mg/ml did not influence the K_m or V_{max} indicated above.

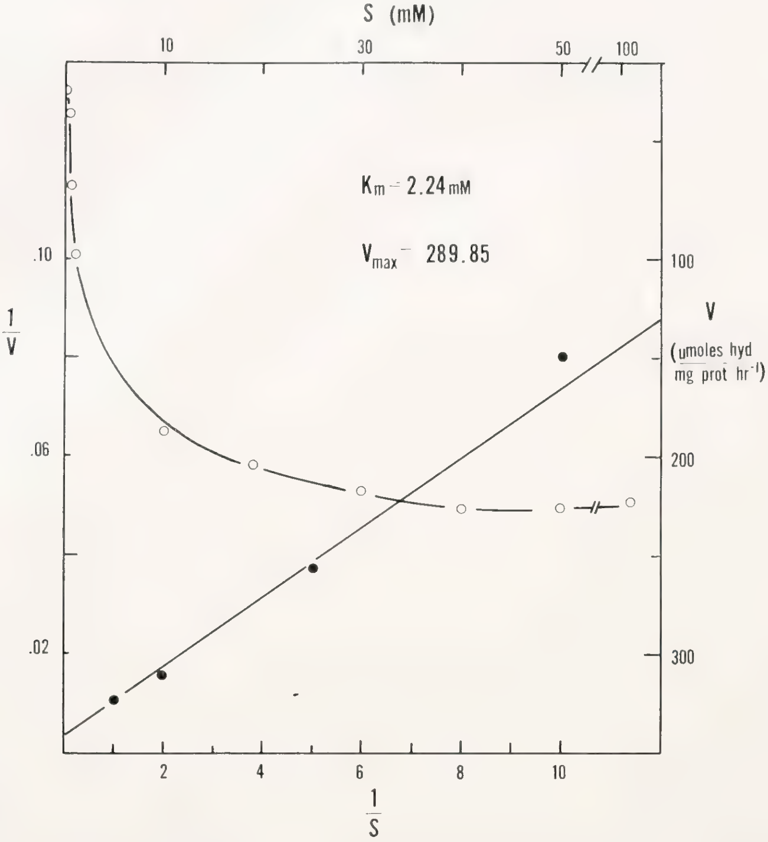

FIGURE 4. Effect of substrate concentration on trehalase activity; incubation mixtures contained in a final volume of 1.225 ml: 100 μmoles citrate buffer at pH 4.6, 0.025 ml enzyme and trehalose in various amounts.

Effect of inhibitors

The effect of metal ions on the hydrolysis of trehalose is presented in Table I. Maximum activity is based the average of three estimations with citrate buffer at pH 4.6. At relatively low concentrations the divalent metals inhibited the enzyme from about 9% to 37%. At much higher concentrations the monovalent metals inhibited the trehalose about 35%. No effect was noted when PCMB was used at 10^{-5} *M*.

Purification of trehalase on DEAE cellulose and enzyme specificity

Figure 5 presents the elution profile form the DEAE cellulose for protein, hydrolytic activity as well as hydrogen ion and Cl⁻ concentration of each fraction.

The stages of the purification presented in the materials and methods section are presented in Table II. With this procedure the enzyme was purified about 80 fold. Examination of the purified enzyme indicated that the pH optimum was the same as that in the crude preparation. Table III presents the specificity of the crude and the purified fraction against various glucose containing substrates indicates that no other carbohydrates were detected in the crude fraction and that no other enzymes were further purified on the DEAE column aside from trehalase.

TABLE II

*Enzyme purification**

Fraction	Volume (ml)	Protein (mg)	Sp. act	Units act	Purification
Crude	70.0	724.5	7.30	5288.0	0
Mn^{++} super	73.5	437.2	14.82	7082.0	2.03
Mn^{++} ppt	70.0	280.4	0.10	28.0	—
30% acet ppt	70.0	259.3	27.00	7001.1	3.70
50% acet ppt	35.0	20.9	0.11	2.3	—
Acet ppt super	65.0	44.7	146.5	6548.6	20.06
DEAE	15.0	38.4	488.0	22,579.2	80.54

* Incubation mixtures in a final volume of 0.325 ml contained: 50 μmoles citrate buffer, pH 4.6, 10 μmoles trehalose and 0.025 ml enzyme. Mixtures were incubated for 15 minutes at 30° and the liberated glucose assayed by glucose oxidase with substrate controls. The alkaline-copper reagent was used in parallel assays.

TABLE III

*Substrate specificity of trehalase**

		μmoles Hydrolyzed mgm protein/hour	
		Crude	Purified
Trehalose	α 1 → 1	7.3	588.0
Kojiobiose	α 1 → 2	0	0
Maltose	α 1 → 4	0.1	0
Isomaltose	α 1 → 6	0	0
Sophorose	β 1 → 2	0	0
Laminaribiose	β 1 → 3	0	0
Cellobiose	β 1 → 4	0	0
Gentiobiose	β 1 → 6	0	0
Sucrose	α 1 → 2	0.8	0
Melibiose	α 1 → 6	0	0
Lactose	β 1 → 4	0	0
Melezitose	α 1 → 3, β 2 → 1	0.1	0
Raffinose	α 1 → 6, α 1 → 2	0	0
α-Methyl-glycoside	α → 1	0	0
Starch	α 1 → 4	0	0
Glycogen	α 1 → 4	0	0

* Incubation mixtures in a final volume of 0.325 ml contained: 50 μmoles citrate buffer, pH 4.6, 10 μmoles substrate, and 0.025 ml enzyme. Mixtures were incubated for 15 minutes at 30° and the liberated glucose assayed by glucose-oxidase with substrate controls.

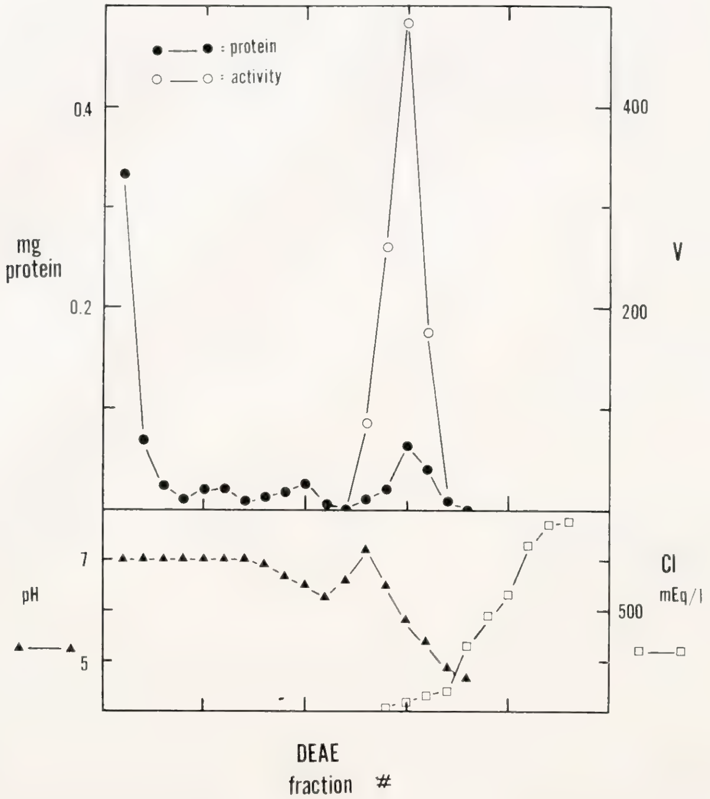

FIGURE 5. Purification of trehalase on DEAE cellulose column. Details of elution are presented in the text.

Energy of activation

The energy of activation of trehalose hydrolysis was determined from the linear portion (0° to 60°) of an Arrhenius plot (log K *vs* 1/T) where the slope is equivalent to -E/2.303R (Fig. 6) and found to be 9321 Cal/mole from duplicate experiments. It was also observed that 86% of the enzymatic activity remained after one hour at 70° providing substrate was present, whereas 40% of the hydrolytic activity was lost in 10 minutes at 70° in the absence of trehalose.

DISCUSSION

During the past ten years considerable interest has been directed toward the study of trehalases from divers sources. A resume of these investigations is presented in Table IV. With the identification of trehalose as the predominant circulating sugar in insects (Wyatt and Kalf, 1957) many investigations have been directed toward the trehalases from this class of arthropods. The further identification of trehalose in a number of invertebrates with representatives from 12 phyla (Fairbairn, 1958) and in a number of protists (Cochrane, 1961; Sussman,

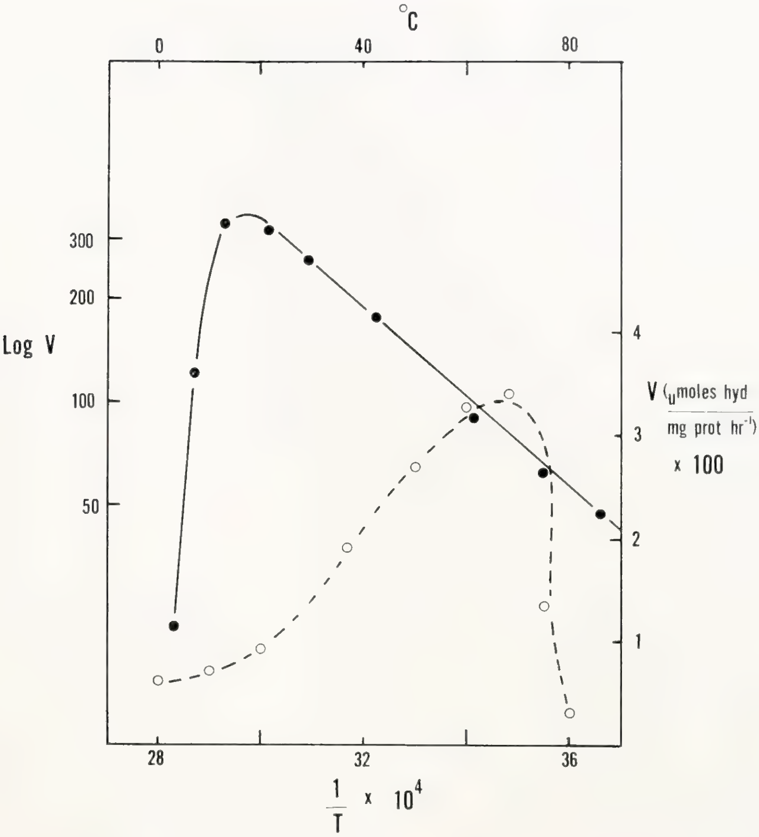

FIGURE 6. Arrhenius Plot (1/T *vs.* log K) of trehalase activity; incubation mixtures contained in a final volume of 0.325 ml: 50 μmoles citrate buffer pH 4.6, 20 μmoles trehalose and 0.025 ml enzyme.

1961; Clegg and Filosa, 1961) has increased the interest in this sugar. It is of considerable importance therefore to have a rapid accurate means of assaying this non-reducing disaccharide. We feel the trehalase isolated from the symbiont of *Blaberus* represents a source for such an analytical enzyme.

The trehalase reported herein has a pH optimum of 4.6 which is somewhat lower than most of the enzymes heretofore reported, one exception being that of the trehalase from *Aspergillus oryzae* reported to have optimum activity at pH 4.0 (Horikoshi and Ikeda, 1966). Courtois, Petek and Kolahi-Zanovzi (1962), also reported that an enzyme from yeast possessed two optima, and at 5.3 and one at 4.1, however, the data presented by those authors did not reveal well defined peaks of hydrolytic activity at either the above hydrogen ion concentrations. The pH optimum and K_m of the trehalase from the symbiont of *Blaberus craniifer* is quite similar to that reported by Gilby, Wyatt and Wyatt (1966) for the midgut trehalase of *Blaberus discoidalis*. Those authors obtained a 100,000 *g* supernatant fraction from homogenates of washed gut tissue. Whether there was any relation-

TABLE IV

Characteristics of some reported trehalases

Source	pH optimum	Km (mM)	Author
Aspergillus oryzae	4.0	2.5	Horikoshi and Ikeda, 1966
Symbiont from *Blaberus craniifer*	4.6	2.2	Fisher and McAlister, present paper
Blaberus discoidalis, midgut	5.0	0.5	Gilby *et al.*, 1966
Bombyx mori, whole pupae	5.2	0.44–.47	Satio, 1966
Yeast	5.3 (4.1)	0.9	Courtois *et al.*, 1962
Bombyx mori, larval midgut	5.4	2.9	Horie, 1959
Melanoplus differentialis, whole adults	5.5	5.1	Derr and Randall, 1966
Neurospora crassa	5.5	0.27 (pool)	Hill and Sussman, 1964
Galleria mellonella, whole larvae	5.5	0.13 sic	Kalf and Rieder, 1958
Dictyostelium discoideum	5.5	1.2	Ceccarini, 1966
Phormia regina, whole adults	5.6	0.67	Friedman, 1960
Saccharomyces cerevisiae	5.7	0.41	Panek and Souza, 1964
Hyalophora cecropia, larval midgut	5.7	0.40	Gussin and Wyatt, 1965
Phormia regina, flt. musc. mito.	5.8	1.3	Hansen, 1966
Blaberus discoidalis, thr. musc. micr.	6.0	3.3	Gilby *et al.*, 1966
Swine Intestine	6.0	3.0	Dahlquist, 1960
Hyalophora cecropia, thr. musc. micr.	6.5	3.6	Gussin and Wyatt, 1965
Streptomyces hygroscopius	6.5	18.0	Hey and Elbein, 1968
Plantago ovata	6.5	0.3	Courtois *et al.*, 1962
Melolontha vulgaris, whole adults	6.5	0.7	Courtois *et al.*, 1962
Hybrid yeast (#12836)	6.9	10.2	Avigad *et al.*, 1965

ship between a bacterial enzyme similar to that described in this paper and the gut trehalase described by Gilby *et al.* (1966) is unknown, but comparative studies on such enzymes would be of interest. Such speculation on the role of symbionts in the enzymology of insect gut has been proposed by Duspiva (1954). It is also of interest that the enzyme described in the present report does not show any inhibition by phosphate or Tris buffers. Such inhibition of trehalases by phosphate buffers has been reported by Hey and Elbein (1968) for the enzyme from *Streptomyces hygroscopius.* Inhibition of trehalases by Tris buffers have been reported by Hey and Elbein (1968), Gilby *et al.* (1966) and Friedman (1960), for enzymes from *S. hygroscopius*, *Blaberus discoidalis* and *Phormia regina.*

The Km for the symbiont enzyme is $2.2 \times 10^{-3} M$ which is markedly similar to most reported trehalases; however that enzyme characteristic is quite different from that reported by Hey and Elbein (1968) and Avigad, Ziv and Neufeld (1965) for the trehalases from *Streptomyces* and a hybrid yeast. The relatively low Km of the enzyme here described is quite advantageous when this enzyme is considered for the analysis of trehalose in biological materials since that sugar often occurs at very low concentrations (Fairbairn, 1958). The energy of activation of this trehalase is about 9300 Cal/mole which is consistant with a large number of carbohydrases, however, it is considerably lower than that reported for the trehalase from *Blaberus discoidalis* muscle microsomes as 15,000 Cal/mole by Gilby *et al.* (1966).

By simple acetone precipitation and passage of the present of enzyme through a DEAE cellulose column the enzyme was purified 80 fold and the specificity for trehalose was maintained. No hydrolysis was observed when 16 other glucose containing oligo- and polysaccharides were examined. The yield of enzyme obtained from symbionts grown on glucose, cellobiose and maltose did not differ significantly from those grown on trehalose; however, the yield of bacterial cells was much less when cellobiose and maltose was used as the only carbon source of interest in this line is the fact that cells grown on maltose and cellobiose did not have any enhanced α 1 → 4 or β 1 → 4 glucosidase activity.

The role of this symbiont in the physiology of *Blaberus* remains obscure. Wyatt (1967) proposed that the trehalase of the insect intestine would prevent the loss of trehalose via the feces since that disaccharide upon entry into the intestine by diffusion would be hydrolyzed to glucose within the gut and again pass into the hemocoel as glucose via diffusion. Again it is not known if the symbiont participates in this function. The trehalase of the symbiont is not an exoenzyme and it would appear that this point would rule out any effect on maintenance of trehalose levels within the insect.

SUMMARY

1. A trehalase from a gram negative symbiont of *Blaberus craniifer* has been isolated and purified 80 fold.

2. Hydrolysis of trehalose was linear with respect to time and protein concentration.

3. The pH optimum of the enzyme was found to be 4.6, the K_m 2.2×10^{-3} M and the V_{max} about 290 μMole trehalose hydrolyzed/mgm protein hr^{-1}.

4. The energy of activation is found to be 9321 Cal/mole and enzymatic hydrolysis was inhibited by monovalent and divalent metal; however, no effect of the sulfhydryl inhibitor PCMB was noted.

5. The merit of this source and this trehalase as an analytic reagent is discussed.

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AN AUTORADIOGRAPHIC ANALYSIS OF MELANOGENESIS IN THE FIDDLER CRAB, *UCA PUGNAX* (S. I. SMITH)

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The epidermis plays a central role in the metabolic activities of crustacea. One facet of these activities involves pigment cells in which two distinct phases of behavior can be distinguished: (1) physiological color change, a rapid mobilization of pigment leading to pigment migration, probably within pre-established cellular channels, and (2), morphological color change, a slower modification of the quantity of pigment per chromatophore or of chromatophore number per unit area of epidermis (Brown, 1934; Green, 1964a, b). An hypothesis (Babak, 1912; Brown, 1934) which closely relates morphological color change to physiological color change may not be valid for crustacean. Specifically, there seems to be no correlation between the state of pigment dispersal within the chromatophore and melanin synthesis (Green, 1964b).

The present study reports the results of autoradiographic analysis of melanin formation in fiddler crab epidermis.

MATERIAL AND METHODS

Autoradiography at the tissue level

Kukita and Fitzpatrick (1955) and Brunet and Small (1959) prepared autoradiographs for a study of melanogenesis from human and mouse tissue. In both cases tissue was incubated in ^{14}C-tyrosine, which is incorporated into insoluble melanin if the melanocytes are active. Both teams of workers reported difficulty in observing exposed silver grains against the background of melanin. This difficulty is partially obviated with fiddler crab tissue because the pigment is periodically distributed into the finest processes of the melanophore. When so distributed the pigment density is such that melanin granules and silver grains can be distinguished. The use of phase contrast optics also heightens the optical disparity between silver grains and melanin granules. Illumination through a trinocular tube of the microscope is reflected from silver grains and provides an additional aid in distinguishing melanin granules and silver grains.

Male specimens of *Uca pugnax* were collected from the salt marsh at Sippewisset, Massachusetts, and brought to the Marine Biological Laboratory, Woods

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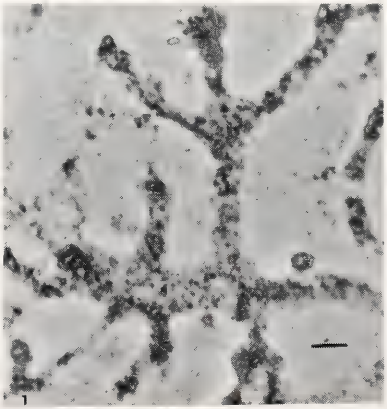
Hole, Massachusetts. The crabs were maintained unfed in a fiberglass tub with perforated floor. Flowing sea water maintained a water level of about $\frac{1}{8}$ inch. Crabs were utilized randomly from this supply.

Entire and destalked animals were injected with 0.05 ml of 2.5 μC 2, 5, -^{3}H-tyrosine in sea water through the arthrodial membrane at the base of the fourth walking leg. All crabs were dechelated to reduce the hemocoel volume, and maintained for two days on a black background to maximize pigment dispersal.

The tissue chosen for examination was the epidermis lining the carapace. This tissue is extremely friable and cannot be stripped from the exoskeleton without considerable destruction and distortion. Consequently the epidermis-exoskeleton complex was fixed *in situ* in San Felice fixative (10% formalin in sea water containing 1% chromic acid plus 10 drops acetic acid). After fixation the hardened epidermis was stripped from the exoskeleton with minimal derangement of the tissue. The epidermis was washed in several changes of distilled water to remove the fixative and free tyrosine.

The epidermis was then spread on gelatin-coated microscope slides (Brunet and Small, 1959). Slides were dipped in Kodak NTB-2 liquid photographic emulsion diluted $\frac{1}{3}$ with distilled water, dried and stored in light-tight boxes in the refrigerator for 35 days. They were developed in Dektol, fixed, washed and



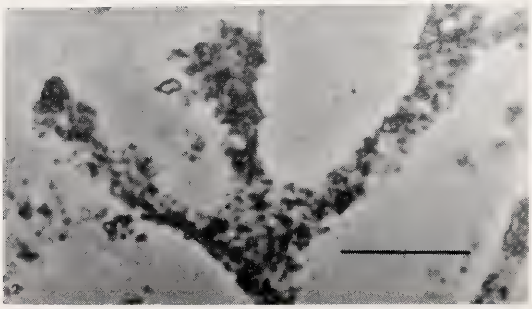


Figure 1. Autoradiograph prepared from a whole-mount of fiddler crab epidermis with dispersed pigment; bar = 50 μ; phase contrast optics.

Figure 2. Autoradiograph prepared from a whole-mount of fiddler crab epidermis; detail of Figure 1, bar = 50 μ; phase contrast optics.

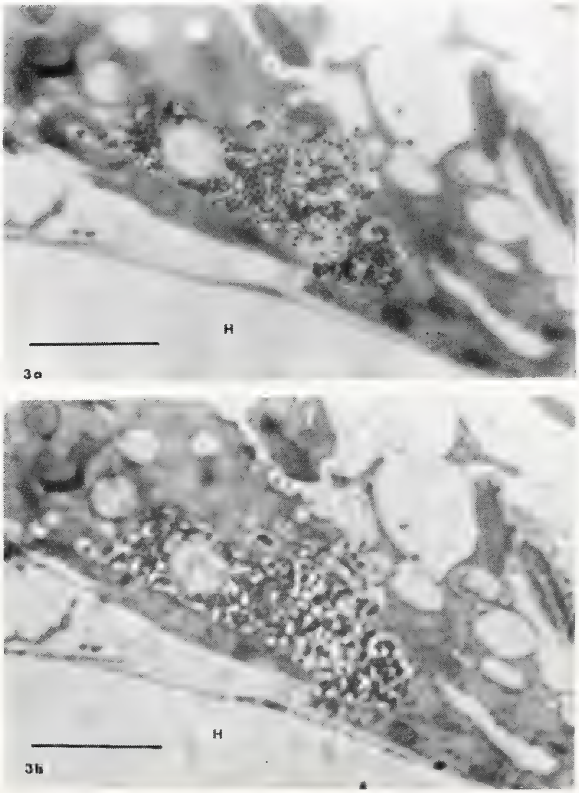


FIGURE 3a. Autoradiograph prepared from 0.5 μ thick section of fiddler crab epidermis, H = hemocoel; phase contrast optics, bar = 20 μ; 3b. As in Figure 3a but epi-illuminated.

dehydrated in a graded series of alcohols. The emulsion-covered tissue was mounted with permount. Observations and photographs were made with Wild phase-optical equipment.

Autoradiography at the cellular level

Additional studies of melanogenesis were made as part of an electron microscope study of the structure of the epidermis. Techniques appropriate for electron microscope observation also yield thick tissue sections suitable for autoradiographic study with the light microscope.

Entire and destalked crabs were each injected with 2.5 μC ^{3}H-tyrosine in 0.05 ml sea water and were maintained as previously described. Crabs destalked two days before injection were maintained for 4 days. The carapace was removed from the crab and freed of adhering tissues (*e.g.,* digestive gland) and was cut into pieces about 5 mm^2. These pieces of exoskeleton with attached epidermis were fixed in 5 ml of 2.5% glutaraldehyde in 0.2 *M* sodium cacodylate buffer containing 0.005 *M* $CaCl_2$ and 0.25 *M* sucrose. After 1.5 hours in the fixative the tissue was washed in 5 ml 0.2 *M* cacodylate buffer for 30 minutes with three changes of buffer. Post-fixation in 1.3% OsO_4 in 0.2 *M* sodium cacodylate buffer for 1 hour was followed by a 30-minute distilled water wash. The preceding steps were carried out at 0° C. with continuous agitation.

Immediately after the final washing, the tissue was peeled from the exoskeleton and was cut into pieces 1 mm^2. Dehydration in a graded series of cold acetone-H_2O mixtures followed. The tissue remained in 100% acetone at room temperature for 8 minutes with two changes.

Infiltration in a graded series of epon-araldite-acetone mixtures to 100% epon-araldite (1.26:1) followed over a three hour period (Voelz and Dworkin 1962). The tissue was flat-embedded in epon-araldite and polymerized at 60° C. for 72 hours.

Sections 0.3–0.5 μ thick were prepared with a Porter-Blum MT-2 ultratome. Sections were floated on water and attached to pre-cleaned microscope slides.

Autoradiographs were prepared from this material as previously described.

RESULTS

Green, 1964b found that *in vivo* incorporation of ^{14}C-tyrosine into melanin was independent of the degree of dispersal of the pigment granules within the melanophore. This does not show that melanin synthesis occurs within the melanophores. The present study was designed to identify the site of melanin synthesis in *Uca pugnax*. Autoradiographs prepared from the epidermis indicate that melanin synthesis occurs only within the melanophore.

Figure 1 is a low power view of epidermis, containing a melanophore with dispersed pigment. A grid was superimposed over this photograph and the silver grains in 20 randomly chosen squares over a melanophore process were counted. Similarly silver grains located in 20 squares which were not over a melanophore process were counted. Squares outside of the boundary of the melanophore had 2.9 ± 0.5 (mean ± S.E.) grains per square as compared with 13.75 ± 0.8 grains per square over a portion of a melanophore. These counts differ significantly ($P < 0.01$—Student's t-test).

Figure 2 is a higher power view of part of Figure 1. Silver grains can be distinguished from melanin granules by their hollow centers. The apparent hollow center of the silver grain is due to its light-reflecting characteristic as opposed to the light-absorbing nature of the melanin granule. This disparity may be heightened by illuminating the section through one of the trinocular barrels of the microscope. With such epi-illumination the silver grains reflect light, whereas the melanin granules remain dull (Fig. 3a, b).

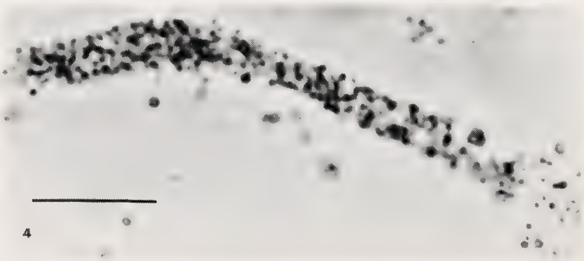
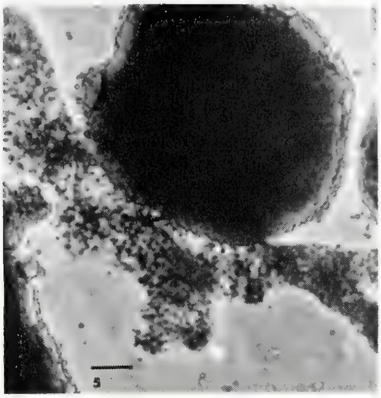


FIGURE 4. Autoradiograph prepared from a whole-mount of fiddler crab epidermis with dispersed pigment, bar = 20 μ.



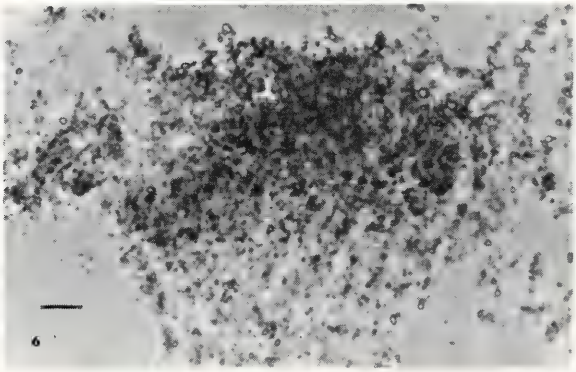


FIGURE 5. Autoradiograph prepared from a whole-mount of destalked fiddler crab epidemis with both dispersed and contracted pigment; upper pigment mass contracted, bar = 20 μ.

FIGURE 6. Autoradiograph prepared from a whole-mount of destalked fiddler crab epidermis. Contracted pigment mass ruptured releasing pigment granules. Lower density of granules enables distinguishing silver grains from melanin granules, bar = 20 μ.

Figure 4 is high-power phase optical view of a melanophore process. This section was analyzed in a fashion similar to Figure 1. Twenty squares not containing any portion of a melanophore had 0.95 ± 0.2 silver grains per square as compared with 4.2 ± 0.4 grains per square over a portion of the melanophore. These grain counts are significantly different ($P < 0.01$).

Crabs whose eye-stalks have been removed have melanin concentrated in the center of the melanophore (Fig. 5). In this condition it is difficult to distinguish silver grains from melanin granules because of the high density of the latter. Only at the edges of the mass of melanin can silver grains be distinguished. In a number of cases preparative treatment of the tissue led to rupture of the concentrated mass of melanin. In these fortuitous cases the density of the melanin granules is reduced and the silver grains can be distinguished (Fig. 6).

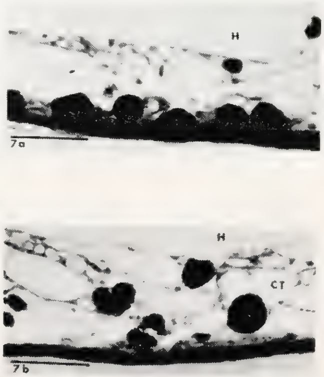
In thick sections prepared as for electron microscopy it can be seen that the melanophores are present on both the environmental and hemocoel surfaces of the epidermis (Fig. 7ab). Figure 8 shows a comparable section with the pigment partially dispersed. Tissue with dispersed pigment is considerably more resistant to damage during processing than is tissue with contracted pigment granules. Apparently the filling of cellular channels with melanin confers considerable strength on the tissue (*cf.* Elias 1942).

Figures 9 (a–c) and 10 show some characteristics of the finer melanophore processes. Figure 9a shows the confluence of melanophore processes, Figure 9b the termination of a process, and Figure 9c the single-file nature of pigment granule migration within a process. Figure 10 shows melanin granules entering (or leaving) a process.

Melanin occurs in the fiddler crab epidermis in approximately spherical granules with a mean diameter of 0.98 μ ± 0.02. In comparison, mammalian melanin granules (melanosomes) are 0.1 μ in length and 0.05 μ in width (Drochmans 1963, 1966). Light microscopy reveals no internal structure within *Uca* melanin granules but studies with the electron microscope are being carried out.

DISCUSSION

Kukita and Fitzpatrick (1955) proposed an autoradiographic assay for mammalian tyrosinase activity based on the enzyme-catalyzed conversion of water-soluble ^{14}C- or ^{3}H-tyrosine to water-insoluble melanin. The theoretical scheme for melanin synthesis involves the enzymatic (tyrosinase) oxidation of L-tyrosine to 3,4-dihy-



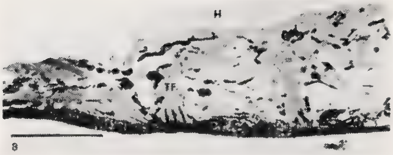


FIGURE 7a. Thin section (0.5 μ) of fiddler crab epidermis with contracted pigment, H = hemocoel bounded by basement membrane, bar = 50 μ; 7b. As in Figure 7a, note presence of melanophores on both epidermal surfaces and also in the spongy sub-epidermal connective tissue (C. T.), bar = 50 μ.

FIGURE 8. Thin section (0.5 μ) of fiddler crab epidermis with partially expanded pigment. Note that there is an indication that the melanophores follow the course of tonofibrillae (TF), bar = 100 μ.

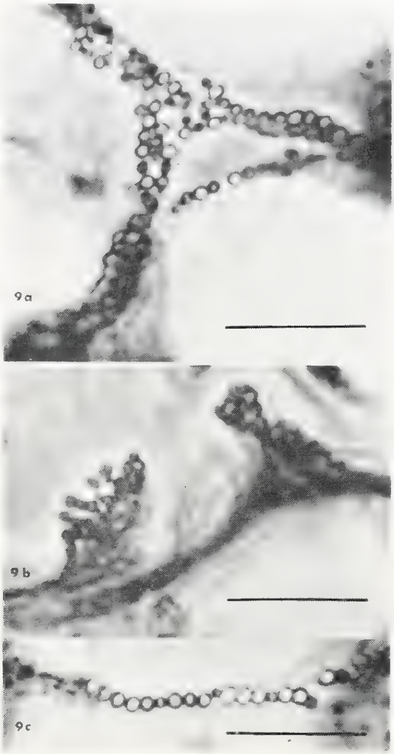


FIGURE 9a. Phase contrast optics, confluence of several melanophore processes, bar = 10 μ; 9b. As in Figure 9a, terminus of melanophore process, bar = 10 μ; 9c. as in Figure 9a, melanin granules in a fine process, bar = 10 μ.

droxyphenylalanine (DOPA) and then to dopaquinone. Dopaquinone undergoes spontaneous oxidation and reduction reactions to yield indole-5,6-quinone. This compound is polymerized to form a large molecule which is thought to be bound through its quinone linkages to the amino or sulfhydral groups of the protein matrix of the pigment granules (Mason, 1959, 1967; Duchon, Fitzpatrick and Seiji, 1968). Nicolaus, Piattelli and Fattorusso (1964) and Nicolaus and Piattelli (1965) (see also Mason, 1967; Duchon *et al.,* 1968) however, hypothesize that melanin is not homopolymeric (a regular polymer of idole-5,6-quinone) but consists of a random polymer (poikilopolymer) of several different monomers including in part, DOPA, indole-5,6-quinone, and 2,3-dihydroxyindole. In either case the product melanin is water-insoluble and has incorporated into it the isotopic label.

Tyrosinase is generally believed to occur in the matrix of the melanosome (Moyer, 1963, Seiji, 1966). Thus the formation of melanin is presumptive evidence for the presence of tyrosinase and melanin granules. In the present work the localization of radioactivity derived from ^{3}H-tyrosine over granules within the melanophore is evidence that melanogenesis occurs only in this cell. Melanogenesis apparently occurs independently of the state of pigment dispersion. The present technique, however, is not sensitive enough to detect short-term differences in the rate of melanin synthesis that may exist between melanophores with expanded

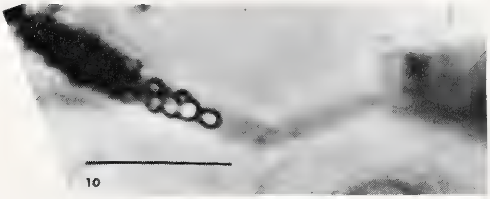


FIGURE 10. As in Figure 9a, melanin granules entering or leaving a process, bar = 10 μ.

pigment and those with contracted pigment. Further work utilizing an *in vitro* assay system will be required to establish this point conclusively.

Seiji, 1966 and Wood and Ingraham, 1965 have suggested as successive layers of melanoprotein accumulate on the melanosome, tyrosinase is inactivated. These workers conclude that there is a reciprocal relationship between degree of melanization and tyrosinase activity within a melanin granule. The present light microscope autoradiographic techniques are not sufficiently sensitive to detect variations in melanogenesis at the sub-cellular level.

The crustacean carapace arises from an epidermal fold of the maxillary segment (Tiegs and Manton, 1958), and thereafter fuses with the united terga of at least 10 body segments behind the head (Snodgrass, 1952). The carapace and its underlying epidermis is folded in the region of the branchial chamber (Travis, 1955, 1957). Figures 7 (ab) and 8 indicate that the epidermis is also double in the region of the back, probably reflecting its origin. As these figures indicate, and as Travis (1955, 1957) showed, for the branchiostegite, the epidermis consists of two layers of cells separated by intra-epidermal (*cf.* subepidermal, Travis, 1955, 1957) connective tissue of a loose spongy type. In *Uca* the connective tissue may be invaded by melanophores (Figs. 7b and 8).

Perkins and Snook (1932) investigated the movement of the red carotenoprotein pigment in chromatophores of the prawn *Palaemonetes*. They found the red pigment to be granular in nature and suggested that the "granules and the protoplasmic medium in which they are carried flow out into collapsed tubes which are the walls of the branched chromatophore, fixed in position, and lying in tissue spaces." Melanin granules in *Uca* are similarly found within chromatophores and seemingly the chromatophores are constructed along the plan proposed by Perkins and Snook. No information is presently available concerning the mechanism of pigment migration in crustacean chromatophores.

I would like to acknowledge the able assistance of Mr. T. Richard Nichols, Miss Mary Rose Neff, and Miss Emily Paynter.

SUMMARY

1. Autoradiographs prepared from whole mounts and sections of *Uca pugnax* epidermis labelled *in vivo* with ^{3}H-tyrosine showed incorporation only into melanin granules within melanophores.

2. Incorporation occurred whether the pigment was in a concentrated (*e.g.*, destalked crabs) or expanded (*e.g.*, entire crabs—black background) condition.

3. Fiddler crab melanin granules appear spherical in light micrographs and have a mean diameter (±S.E.) of 0.98 μ ±0.02.

4. The epidermis of the back of the fiddler crab shows two layers of melanophores, one lining the hemocoel basement membrane, the other lining the exoskeleton. This may be a result of the formation of the carapace as a fold from the maxillary segment. Melanophores and melanophore processes also are found in the spongy intra-epidermal connective tissue.

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THE DEVELOPMENT OF THE ANDROGENIC GLANDS OF A PROTANDRIC SHRIMP [1]

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The protandric nature of the pandalid shrimps from the northwest coast of North America was first observed by Berkeley (1929, 1930). In these species of caridean decapods, the male stages possess hermaphroditic gonads, but only the testicular elements become functional. After one to three years, depending on the species of shrimp, the males transform into fully functional females with true ovaries. Charniaux-Cotton (1958) and Carlisle (1959a, 1959b, 1959c) reported that the onset of the female phase in *Pandalus borealis* coincides with the disappearance of a pair of endocrine organs, the androgenic glands. The role of the androgenic glands in talitrid amphipods is to control primary and secondary sexual differentiation and development through the secretion of an androgenic hormone (Charniaux-Cotton, 1952, 1954, 1955, 1957). Although data have been reported on the androgenic glands of the higher Crustacea (Charniaux-Cotton, Zerbib and Meusy, 1966), little is known concerning the development and differentiation of the androgenic glands of protandric decapods. One reason for this lack of information is the difficulty encountered in obtaining all of the developmental stages of a protandric shrimp on a seasonal basis.

The present report includes most of the developmental stages of the androgenic glands of the Pacific pandalid, *Pandalus platyceros* Brandt. In addition to the analysis of the development of the androgenic glands, correlations are made between structure and function.

MATERIALS AND METHODS

Pandalus platyceros Brandt was collected on a monthly basis from June 1965 through January 1968. The shrimp were collected in Lopez Sound, Washington in fifteen to twenty-seven fathoms of water with a ten foot beam trawl. In the laboratory the shrimp were maintained in twenty to thirty gallon plexiglas aquaria that were supplied with running sea water. Generally the shrimp were sacrificed within a day or two of collecting. About 250 animals were utilized in this study.

The abdomen was excised with a razor blade and the carapace of the thorax was cut away to expose the gonads and sperm ducts. The ejaculatory bulbs and associated androgenic glands are located in the region where the sperm ducts are inserted on the sternal portion of the fifth walking legs. The androgenic glands were removed while still attached to the sperm ducts with fine forceps and iridectomy scissors and were immediately placed in fixatives.

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Although Bouin's and Heidenhain's "Susa" fixatives were used initially for orientation purposes, a glutaraldehyde fixation was employed routinely thereafter. Primary fixation was in equal volumes of 5% aq solution of glutaraldehyde in 0.27 *M* NaCl and 0.4 *M* Millonig's phosphate buffer pH 7.4. After one hour fixation the tissue was rinsed in a solution of the phosphate buffer and 0.6 *M* NaCl (1:1 v/v). The tissue was then postfixed in an ice bath for one hour in the following solution: 1 part 4% osmium tetroxide; 1 part phosphate buffer; 2 parts 0.75 *M* NaCl. The osmolarity of all the fixatives and rinse was maintained at about 975 milliosmoles.

The tissue was dehydrated through increasing concentrations of ethanol and passed through three changes of propylene oxide to prepare the tissue for infiltration with Epon epoxy resin. It was then embedded in the resin according to the method of Luft(1961). Sections of one to two microns were cut on a Porter-Blum Ultratome using glass knives. The sections were stained on a hot plate using Richardson's stain (Richardson, Jarett and Finke, 1960), allowed to dry and then covered with Permount or Xam.

RESULTS

The androgenic glands of *Pandalus platyceros* are located on the dorso-medial surfaces of the ejaculatory bulbs which in turn are at the distal ends of the sperm ducts (Fig. 1). The ejaculatory bulbs are highly muscular sphincters that allow the passage of the sperm strand through the male gonopore during copulation. The glands are composed of highly folded anastomosing cords of epithelial cells (Fig. 2). During the male phase each gland has a compact leaf-like appearance with the widest portion resting on the ejaculatory bulb and then extending up to the medial side of the sperm duct. From late spring through early summer the glands reach their maximum size of 1–2 mm in total length. The glands are supported on the sperm ducts by an abundance of connective tissue and small muscle filaments which appear to be derived from the musculature of the ejaculatory bulbs. No associated masses of androgenic tissue was found. Adipose tissue and lymphogenous nodules are located close to the androgenic glands. In paraffin embedded tissue the lymphogenous nodules may be confused with androgenic gland because of their similarity. However, from thick plastic sections and from numerous observations made from lymphogenous tissue in other regions of the shrimp, it is quite evident that this tissue is not androgenic gland tissue.

The early development and origin of the androgenic glands

The developmental state of the androgenic glands of *Panadalus platyceros* is a function of the size of the shrimp and not its age. Shrimp of the same age may vary considerably in size and state of development. In immature shrimp less than 2.2 cm CL (carapace length) which are six to eight months old (September through November), the muscular sheaths that encapsulate the epithelium of the sperm ducts have yet to be formed or are in the initial stages of being formed. A mass

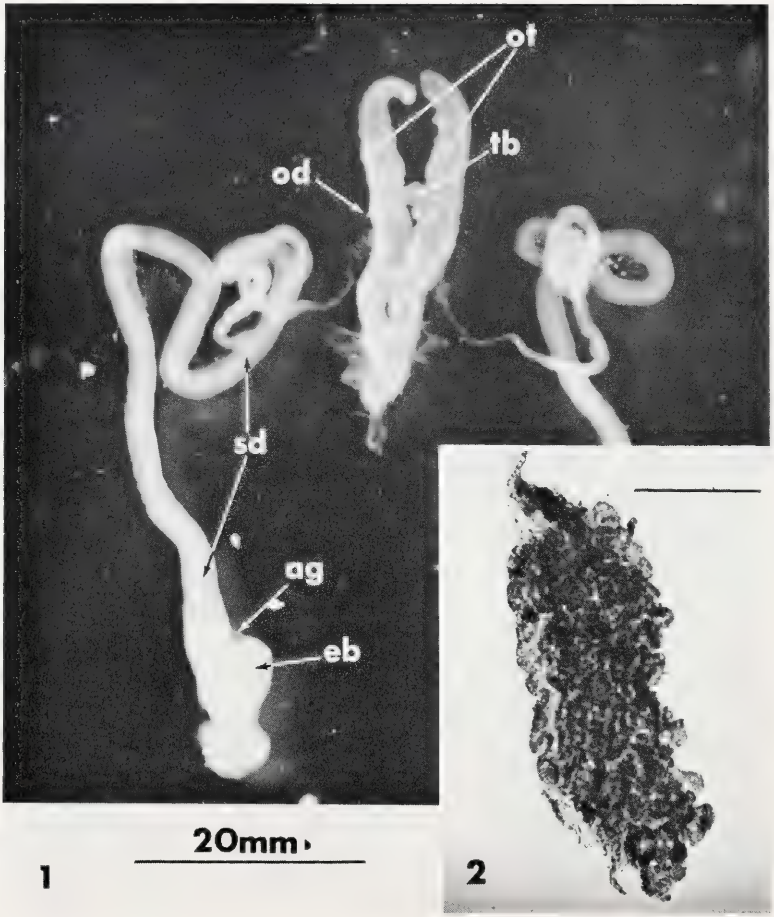
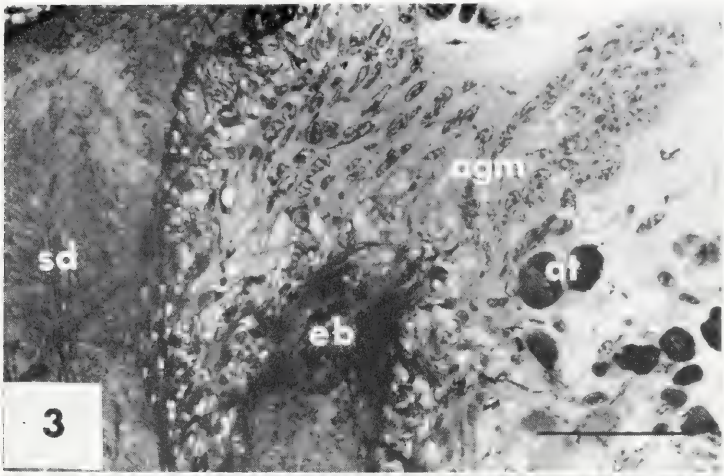


Figure 1. Reproductive system of a male *Pandalus platyceros* (3.5 cm carapace length, August 1967); Whole preparation in sea water: ag, androgenic gland, eb, ejaculatory bulb of sperm duct; od, oviduct; ot, ovotestis; sd, sperm duct; tb, transverse bridge of ovotestis.

Figure 2. Whole mount of an androgenic gland of a male *Pandalus platyceros* (2.9 cm carapace length, June 1967). The slash mark represents 0.5 mm.

of cells can be seen at the dorsal surface of each ejaculatory bulb (Fig. 3), and appear to be the early androgenic glands. In fact, peripheral portions of these cell masses can be seen in cord-like arrays. In immature males larger than 2.2 cm CL, the androgenic glands resemble the adult glands, but they are composed of very few cords of cells. The epithelial linings of the sperm ducts have now developed a muscular sheath. From transverse sections through the distal ends of the differentiating ejaculatory bulbs, it is apparent that androgenic gland cells are proliferating from cells in the ejaculatory bulb epithelium (Fig. 4). These newly formed rectilinear arrays of tissue have moved between the muscle masses that encapsulate this region (Figs. 5 and 6). No mitotic figures are evident in this intermuscular androgenic tissue. The most peripheral muscle bands of the ejacula-



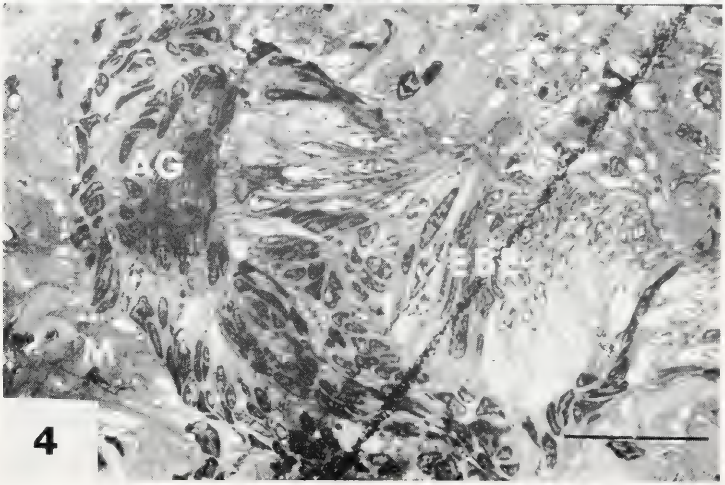


FIGURE 3. Transverse section (1 μ) of the androgenic gland mass from an immature male *P. platyceros* (1.7 cm carapace length, September); Glutaraldehyde-Osmium fixation, Richardson's stain. The slash mark represents 50 microns; agm, androgenic gland mass; at, adipose tissue; eb, ejaculatory bulb epithelium; sd, sperm duct epithelium.

FIGURE 4. Transverse section (1 μ) through the dorsal region of the ejaculatory bulb of an immature male (2.2 cm carapace length, November) showing the relationship between the androgenic gland tissue (AG) and the epithelium of the ejaculatory bulb (EBE); Glutaraldehyde-Osmium fixation, Richardson's stain. The slash mark represents 25 microns, M, musculature of the ejaculatory bulb.

tory bulb appear to disassociate from each other, liberating the glandular tissue into the hemocoel. This phenomenon may account for the muscle bands that are found in loose association with some of the androgenic cords in the adult male stage.

Although the androgenic gland cells appear to originate from the differentiating epithelium of the ejaculatory bulb, actual cell division and active growth occur during the winter and spring months in the androgenic cords that lie free in the hemocoel.

In reproductive males that have sperm ducts greatly distended with sperm, the sperm duct epithelium produces a secretion stained with Orange G that forms the sheath of the sperm strand (Fig. 7). At this stage of development no intermuscular masses of androgenic tissue are evident within the musculature of the ejaculatory bulb, and the androgenic glands have lost their connection with the musculature. In contrast, males which have not produced sperm during the reproductive season still show intermuscular masses of androgenic tissue (Fig. 6) and the androgenic glands are connected with the musculature of the sperm ducts.

Carlisle (1959a) reports the androgenic glands of *Pandalus borealis* attached to a strip of vas deferens (sperm duct) epithelium. It is difficult to state for certain for his drawings of this connection are rather diagrammatic, but this epithelial layer may be part of the ejaculatory bulb. Charniaux-Cotton *et al.* (1966) have not seen this connection in the same species of shrimp. These two investigators may be reconciled if one considers that the glands of *P. platyceros* lose their connection with the musculature during the sexually active male stage. Thus the investigators may be describing different stages of development.

There is a striking difference in the morphology of the sperm duct epithelium between reproductive and nonreproductive males. In nonreproductive males, the lumina of the sperm ducts may or may not have become patent and the epithelial layer contains tall columnal, highly compact epithelial cells (Fig. 8). However, in reproductive males, the height of the epithelium has been reduced and its cytoplasmic affinities for basic stains have nearly disappeared (Fig. 7).

The developmental cytology of the androgenic gland cells

The androgenic gland cells of *Pandalus platyceros* generally contain ovoid to discoidal nuclei measuring on the average fifteen microns along their long axis. The shape of the nucleus is related to the number of cells per unit length of androgenic cord. When the number of cells per unit length is small, the nuclei tend to be more ovoid in appearance. When the number of cells per unit length is large, the cells are highly compressed against each other very much like overlapping shingles. This results in extremely flat, discoidal nuclei. The general irregular outline of the intermuscular androgenic gland cell nucleus probably is due to mechanical pressure from the muscle bands. The cytoplasm of the gland cells generally has affinities for basic stains, although there is considerable variation in stainability from cell to cell. No histologically stainable cell product is ever evident in any of the cells, although acid phosphatase positive granules (Gomori, 1950) are present in many of the cells.

The developmental histology of the androgenic cells of *P. platyceros* can be divided into six discreet stages (Fig. 9).

Stage 1 or the stage of proliferation is typical of immature androgenic cord cells and intermuscular cells. This stage is characterized by cells with relatively small cytoplasmic volume, small irregularly shaped nuclei with a diameter of about ten microns and, in the cord cells only, an abundance of mitotic figures.

Stage 2 or the stage of cytoplasmic hypertrophy is found during the winter, spring and early summer months at the time of the active male phase. During these times of the year the gonads are actively proliferating spermatogonia and a relatively small number of oogonia. This stage is characterized by an elongation of the long axis of the cells to 50 μ in length; a flattening and lengthening of the nuclei into ovoids or discs; the presence of dense chromatin granules and ribbons in the nucleoplasm; an increase in the affinity of the cytoplasm for basic stains; an increase in cytoplasmic volume; the presence in the cytoplasm of Gormori acid phosphatase positive granules; and a thinning of the androgenic sheath to a thickness of one or two microns. This stage of development is characteristic of most of the cells that comprise the androgenic glands proper and may be considered to represent the typical androgenic gland cells of *Pandalus platyceros*.

Stage 3 or the stage of vacuolization is found during the winter, spring and early summer months at the time of the active male phase, but the total number of vacuolated cells comprise only a small number of the total number of gland cells. This stage appears to reach a peak during the early summer months. They are not localized in any specific region of the gland but are scattered in clusters throughout the gland. The morphological characteristics of this stage are the gradual loss of affinity by the nuclei for basic stains and the swelling of the nuclei into ovoid form; the loss of affinity of the cytoplasm for basic stains; the disappearance of the Gomori acid phosphatase positive granules from the cytoplasm; and the formation in the cytoplasm of vacuoles which do not stain in any characteristic way.

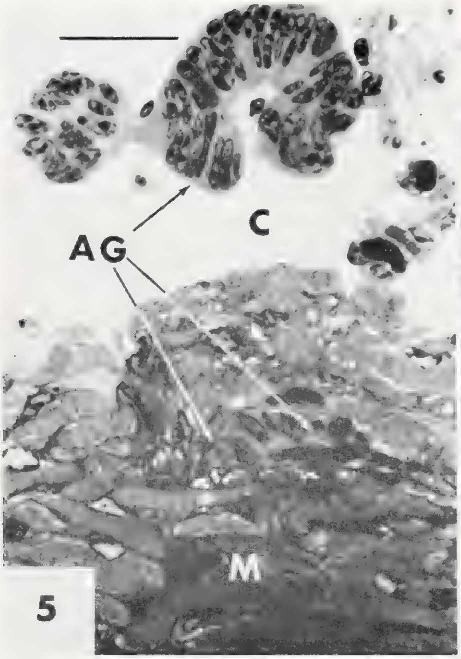
Stage 4 or the stage of cellular breakdown closely follows the preceding stage and is always found in association with stage 3. This stage is characterized by enlarged nuclei that have almost completely lost their affinity for basic stains, and by the total degeneration or break-up of the cytoplasm. At times, such large swollen nuclei can be found intact in the hemocoel near the androgenic cords.

Stage 5 or the stage of transitional atrophy is found in all of the androgenic gland cells of transitional males during the late summer and early autumn months. Also such cells are found in the peripheral regions of the androgenic glands of nontransforming (nonreproductive) males during the same times of the year. This stage is characterized by irregular and densely staining pycnotic nuclei, and diminution of the long axis of the cells, the presence of large cytoplasmic inclusions that range up to six microns in length, and a thickening and folding of the androgenic sheath.

Stage 6 or the ghost stage is characterized by empty sheaths of androgenic cords. It occurs in the latter months of autmn and early winter. The entire androgenic glands of transforming males is typified by this stage. The gonads of these shrimp totally lack testicular elements and contain only ovarian cells. Androgenic glands of nontransforming males contain many ghost areas in the periphery of the gland, but also show new cords regenerating from the intermuscular cells (stage 1). This stage is characterized by empty sheaths of androgenic cords or "ghosts" of androgenic gland cells in which pycnotic nuclei are sometimes evident.

DISCUSSION

The observations that the androgenic glands of the protandric hermaphrodite, *Pandalus platyceros* originate from the epithelium of the sperm duct that will differentiate into the ejaculatory bulb corroborate the results of previous workers who have given a genital origin for the androgenic glands of the higher Crustacea (Charniaux-Cotton *et al.*, 1966). The sperm ducts and associated ejaculatory bulbs originate from the posterior extensions of the early gonadal tube (Hoffman, 1968). Any derivative from the epithelium that lines these tubes would be of gonadal origin. Demeusy (1960) noted in the postembryonic development of the androgenic glands



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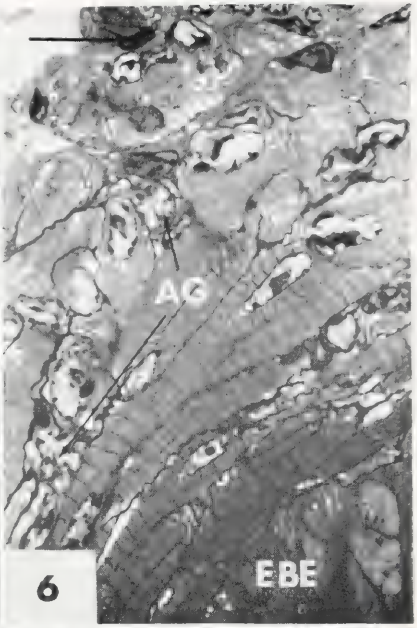

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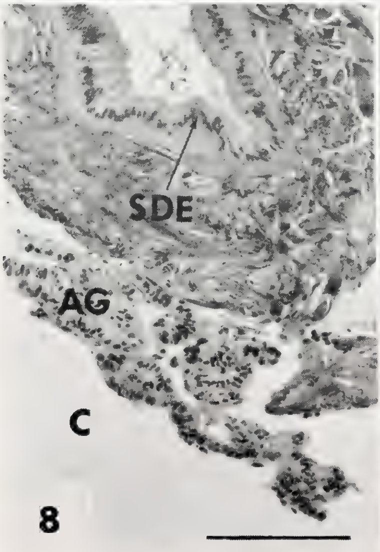

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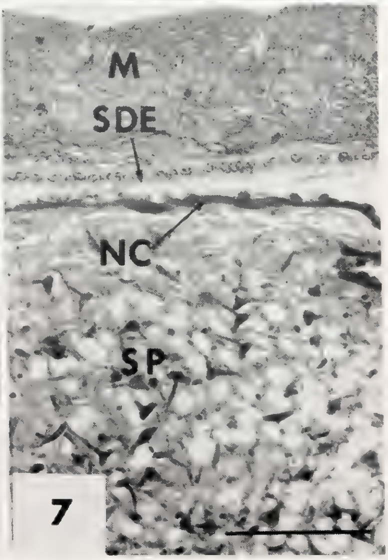

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FIGURE 5. Transverse section (1 μ) through the musculature of the ejaculatory bulb showing androgenic gland cells between the muscle bands (2.4 cm carapace length, December); Glutaraldehyde-Osmium fixation, Richardson's stain. The slash mark represents 25 microns; AG, androgenic gland cells; C, hemocoel; M, musculature of the ejaculatory bulb.

FIGURE 6. Transverse section (1 μ) through the musculature of the ejaculatory bulb showing androgenic gland cells between the muscle bands (nonreproductive male, 3.0 cm

of *Carcinus maenas* that small intermuscular masses of androgenic tissue are present by the sperm ducts, but these masses were not related to the epithelium of the sperm ducts proper.

It is apparent that the androgenic glands of this protandric shrimp disappear because the source of these cells has completely differentiated into an ejaculatory bulb epithelium. Therefore this epithelial layer has lost the ability to differentiate into androgenic gland cells.

Following spermiogenesis, when the sperm ducts have become greatly distended with sperm and spermatic fluid, the tall columnar epithelial cells of the sperm ducts secrete a sheath that forms the sperm into long transparent strands or filaments. These epithelial cells then appear to regress, but do not completely degenerate until several months later. This change in function and morphology coincides with the disappearance of the intermuscular androgenic gland cells. At this time, the epithelium may have lost its capacity to differentiate into androgenic gland cells and sperm sheath producing cells. Before sperm was present in the ducts, the epithelium was in an "undifferentiated" state. In a study of the reproductive cycle of *Pandalus platyceros,* it has been shown that most if not all the males that become reproductive generally transform into females, while those that do not become reproductive generally remain in the male phase for another year (Hoffman, 1968). They then become reproductive and soon afterward transform into females.

The rectilinear arrays of androgenic tissue between the muscle bands of the differentiating ejaculatory bulbs may explain the morphology of the adult glands. As young cords of tissue work their way out of their muscular enclosure, cell divisions become evident. At this time the cells grow in length and become associated with each other into discreet glands. The gland proper is attached to the dorsal surface of each ejaculatory bulb during the male phase. It appears then that the replacement zone is located in this region of the sperm duct. Numerous observations show that the intermuscular androgenic masses find passage out of the muscle bands that envelope this region of the duct, but it is not known what physiological or mechanical mechanisms occur in the muscles to permit this release. Since numerous loose muscle bands are sometimes evident in or alongside the androgenic glands, they are thought to originate from the musculature of the ejaculatory bulb.

The androgenic glands have been reported to secrete in a holocrine manner (Charniaux-Cotton, 1960; Charniaux-Cotton and Kleinholz, 1963; Tcholakian and Reichard, 1964) on the basis of observations on the number of degenerating regions of the glands at times when they are considered to be functional. Yet no histological or histochemical products have ever been reported in any androgenic gland cell (Charniaux-Cotton *et al.,* 1966). No secretory product appears to be present in

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carapace length, August); Glutaraldehyde-Osmium fixation, Richardson's stain. The slash mark represents 10 microns; AG, androgenic gland cells; EBE, ejaculatory bulb epithelium.

FIGURE 7. Transverse paraffin section (6 μ) through the sperm duct of a postreproductive transforming male (3.6 cm carapace length, November), Hematoxylin-Halmi stain. The slash mark represents 50 microns; M, musculature of sperm duct; NC, noncellular sheath of sperm strand; SDE, sperm duct epithelium; SP, sperm.

FIGURE 8. Transverse paraffin section (6 μ) through the sperm duct and attached androgenic gland (2.8 cm carapace length, July), Hematoxylin-Halmi stain. The slash mark represents 100 microns; AG, androgenic gland; C, hemocoel; SDE, sperm duct epithelium.

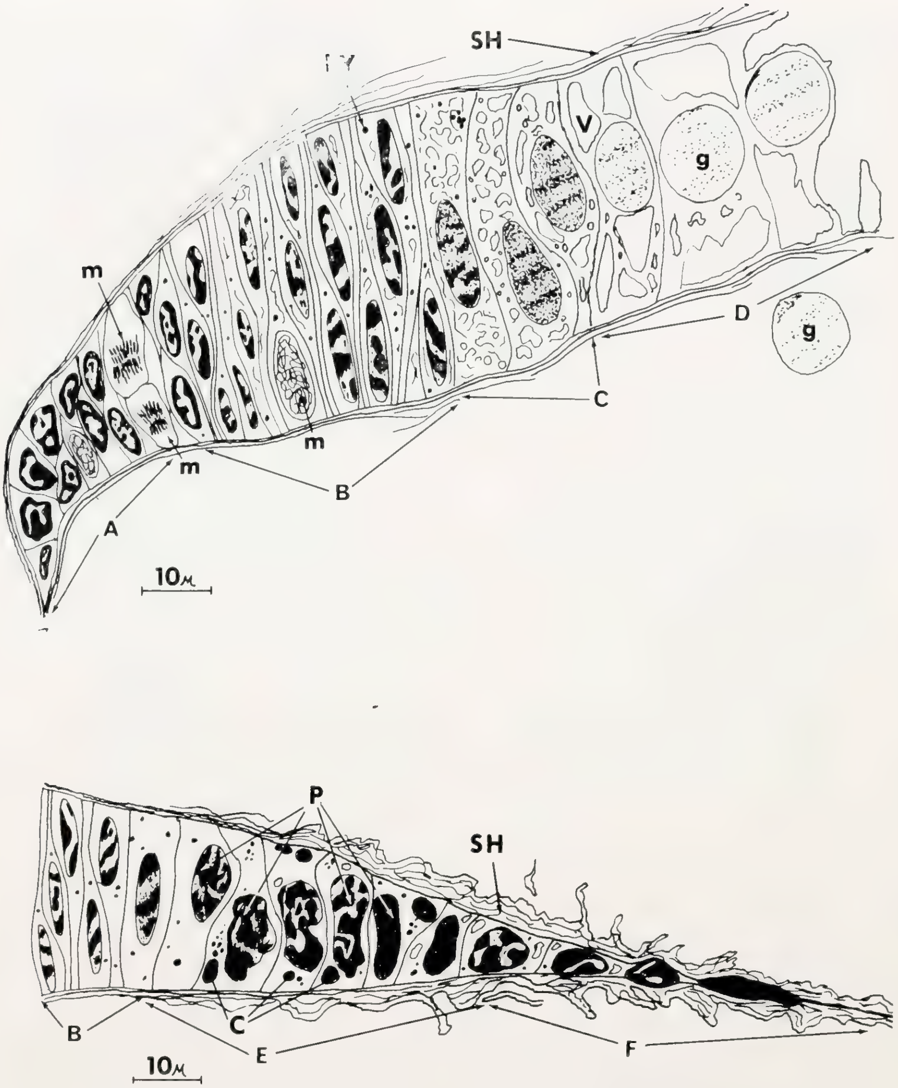

FIGURE 9. The developmental stages of the androgenic gland cells of *Pandalus platyceros*, A. Stage 1 or the stage of proliferation; B. Stage 2 or the stage of cytoplasmic hypertrophy; C. Stage 3 or the stage of vacuolization; D. Stage 4 or the stage of cellular breakdown; E. Stage 5 or the stage of transitional atrophy; F. Stage 6 or the ghost stage. C, cytolysosomes of Novikoff?; g, giant nuclei; Ly lysosomes?; m, mitotic nuclei; P. pycnotic nuclei; SH, sheath of androgenic cords.

any of the androgenic gland cells of *P. platyceros*. King (1964) has described multivesicular bodies in the androgenic gland cells of *Pachygrapsus crassipes* and these Gomori acid phosphatase bodies appear to be lysosomes. Similar inclusions have been found in the gland cells of *P. platyceros*, especially in stage 2 but absent in subsequent stages. Novikoff (1961) has stated that lysosomes are not the causative agents of cell death, but become activated releasing their autolytic enzymes after the cell has already died. Perhaps the stage 3 cells are in the initial stages of cell death since the lysosome-like bodies are no longer evident.

The degenerating androgenic glands of transforming *P. platyceros* males do not at all resemble the degenerative cells described by Charnaiux-Cotton (1960) as representative of holocrine secretion. No vacuolar cells are present in the glands during the transformation to the female phase. The nuclei do become pycnotic, but the cytoplasm stains intensely with basic stains and numerous large inclusions are evident within the cells. These large inclusions so typical of stage 5 may be similar to the large lysosomes or cytolysosomes described by Novikoff (1959, 1960) in dying liver cells and dying cells of atretic ovarian follicles. Scharrer (1966) has reported large irregular membrane bound bodies up to 4 μ in diameter within the degenerating prothoracic gland cells of *Leucophaea* and *Blaberus* a few days after the final molt. Scharrer believes that these bodies represent autophagic vacuoles.

Further evidence opposing or questioning holocrine secretion as the cause of the degeneration seen in stage 5 is found in the gonad, the major target organ of the androgenic hormone. When the cells are at stages 5 and 6 in late summer and early autumn, the gonad has ceased to elaborate new spermatogonia from the germinal epithelium. By November the androgenic glands have disappeared in transforming males and the gonad has become a true ovary (Hoffman, 1968). The disappearance of the androgenic glands of *Pandalus platyceros* and thus the initiation of the female phase appear to be under some form of inhibitory control from the eyestalk complex (Hoffman, 1968b).

The development of the androgenic gland cells of *P. platyceros* may demonstrate merocrine as well as holocrine activity. In this case the stage 2 cells could be releasing their product in a merocrine manner. The degeneration that is evident in stages 3 and 4 then would be indicative of cell death. In addition nothing significant is known concerning the precise chemical nature of the androgenic hormone due to the lack of a reliable bioassay. Therefore, the mechanism of secretion of the androgenic hormone remains obscure despite previous claims of holocrine activity.

I wish to thank the director of the Friday Harbor Laboratories, Dr. Robert L. Fernald, for the generous facilities afforded to me while this study was undertaken. Also many sincere thanks to Dr. Paul L. Illg for his patience, advice and criticism over the years of this study.

SUMMARY

1. The androgenic glands of the protandric shrimp, *Pandalus platyceros,* are shown to originate from a region of the sperm duct epithelium that will later differentiate into the ejaculatory bulbs.

2. The disappearance of the androgenic glands at the time of sex transformation appear to be related to gonadal function and the secretory activity of the sperm duct epithelium.

3. A developmental scheme is presented for the androgenic gland cells of *P. platyceros.* The cells are classified into six developmental stages.

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MECHANISM OF STARFISH SPAWNING. II. SOME ASPECTS OF ACTION OF A NEURAL SUBSTANCE OBTAINED FROM RADIAL NERVE

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That starfish spawning is induced by injecting a water extract of radial nerves into the coelomic cavity has been well established in about twenty starfish species (Chaet and McConnaughy, 1959; Chaet, 1964a, 1966b, 1967; Noumura and Kanatani, 1962; Unger, 1962). The induced spawning is due to the action of a substance, a polypeptide (Kanatani and Noumura, 1962; Kanatani, 1967; Chaet, 1964b, 1966a, b, 1967), which has been called a "gamete-shedding substance." Efforts to elucidate the mechanism of starfish spawning have been continued in our laboratory in an attempt to know the mode of action of such a polypeptide, which is probably a product of neurosecretion. In a preceding paper of this series (Kanatani and Ohguri, 1966) it was shown that the quantitative distribution of the active substance seems to correspond to the quantity of nervous tissue present in a given part of the body; it occurs also in the tube feet, body wall and cardiac stomach and its quantity is uniform in various parts of the nerve ring-radial nerve system (per dry weight of lyophilized nerve material). It has been further demonstrated that the substance occurs in the coelomic fluid only when starfish are undergoing natural spawning.

The present report deals with the results of some *in vitro* as well as *in vivo* experiments carried out with respect to the action of the active substance obtained from radial nerves on discharge of eggs from the ovary. The effect on spawning of deficiency of bivalent cations in the sea water was also examined, since the preliminary experiments suggest that dissolution of an intercellular cementing substance among the oocytes is a prerequisite for starfish spawning. Further, the relation between the contraction of the ovary and the action of the nerve extract was investigated, since Chaet (1966a) has postulated that the shedding substance contains a "contraction factor." The effect of nerve extract on spawning when it is applied from outside the body was also examined, in order to verify the notion that this neural substance is a neurosecretory substance but not a kind of pheromone which acts among individuals. Some of the results presented here have already been reported in a brief preliminary form (Kanatani, 1964, 1967).

MATERIALS AND METHODS

The material mainly used was the starfish, *Asterias amurensis*. *Asterina pectinifera* was also used in some experiments. The method for obtaining radial nerves was the same as that described in a preceding paper (Kanatani and Ohguri, 1966).

To make the nerve extract, lyophilized radial nerves were homogenized in a small amount of deionized water, and centrifuged at 27,000 *g* or 60,000 *g* for 1 hour. Sodium chloride solution was added to the supernatant to make an isotonic nerve extract (final concentration, 5/9 *M*), which served as the original extract. For the experiments, the original extract was diluted with sea water to appropriate concentrations. The concentration of nerve extract was expressed as the original weight of lyophilized nerve per milliliter of the test solution.

Salt-free nerve extract was prepared as follows. Lyophilized nerve was homogenized in deionized water (40 mg/ml) and centrifuged at 60,000 *g* for 50 minutes. The supernatant (5.8 ml) was gel-filtrated on a Sephadex G-25 column (2.5 × 94 cm) in deionized water. The fractions were assayed with ovarian fragments placed in 5 ml of sea water containing 0.01 ml of the fraction to be tested. The fractions found to be active were pooled and lyophilized. This sample, dissolved in a small amount of deionized water, served as the original salt-free nerve extract.

When nerve extract was to be injected into the coelomic cavity, sodium chloride solution was added to the supernatant of the nerve extract to bring its concentration to 5/9 *M*.

For *in vitro* assay, ovaries were isolated, thoroughly rinsed with sea water, and cut into pieces, 1 to 1.5 cm in length, which were used in the manner described in the preceding study (Kanatani and Ohguri, 1966).

To assay the nerve extract for its contraction-inducing ability, the method of Gaddum (1953) was adopted, using de Jalon's solution with 0.5 m*M* magnesium chloride. An isolated rat uterus, suspended in air, was connected with a kymographic apparatus and the degree of its contraction was recorded when a given test solution was applied to the surface of the uterus. The bath temperature was 31° C. The uterus used was obtained from a proestrus rat of the Wistar strain. Oxytocin solution (Syntocinon, Sandoz, Lot No. 64027; 5 I. U./ml) was diluted and used as the reference standard.

For the *in vitro* experiments, van't Hoff's artificial sea water was prepared by mixing isotonic stock solutions of the component salts. The pH of the artificial sea water was adjusted to 8.3 with 0.05% $NaHCO_3$ and a small amount of 0.1 *N* sodium hydroxide.

For histological study, ovarian fragments ligated at their cut surfaces were fixed in Zenker's (formol) solution and embedded in paraffin. Sections were cut at 5 μ, and stained with azocarmine G, anilin blue and orange G.

RESULTS

Effect of nerve extract on spawning in vitro

Some experiments were conducted *in vitro* in order to determine the mode of action of the nerve extract on the ovary of *Asterias amurensis* with respect to spawning.

The distal part of an isolated ovary was immersed in sea water containing nerve extract (500 μg. lyophilized nerve/ml), and its proximal part in sea water (Fig. 1A). After 1 hour the ovary was thoroughly rinsed with sea water (Fig. 1B) and its gonadal wall was torn with fine forceps to make several small slits, proximally, centrally, and at the proximal part of the treated distal region.

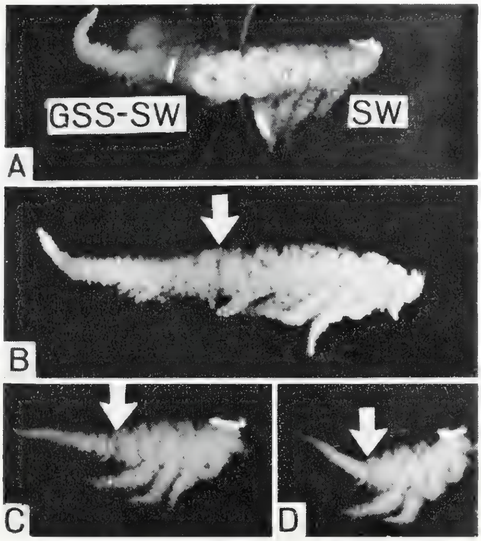

FIGURE. 1. Local treatment of a whole ovary with sea water containing nerve extract in *Asterias amurensis*; (A) an isolated ovary partially exposed to nerve extract and sea water; GSS-SW, sea water containing nerve extract; SW, sea water; (B) same ovary rinsed with sea water immediately after such treatment for 1 hour; arrow, boundary between treated and untreated regions; (C) Same ovary after discharge of eggs from treated portion; (D) same ovary after additional treatment with potassium chloride; B, C, D, same magnification.

Eggs were released immediately from these openings, but discharge of eggs from the regions not treated with nerve extract soon ceased. On the contrary, the shedding of eggs from the slits made in the portion treated with nerve extract was intense and continued so that the treated portion was clearly distinguishable from the nontreated portion. Figure 1C shows such an ovary 50 minutes after the slits were made. When these ovaries were again transferred to sea water containing potassium chloride at a high concentration (sea water 300 ml + 5/9 M KCl 60 ml), eggs were again discharged from the slits. The degree of discharge from the treated portion was very intense in contrast to the nontreated portion. Figure 1D shows the ovary 10 minutes after such treatment with potassium chloride.

Microscopic observation of the released eggs at the time of discharge revealed that those from the portion treated with nerve extract were surrounded only by jelly, whereas, those from the nontreated portion had follicles. Also, breakdown of the germinal vesicles was seen only in oocytes obtained from the treated portion. This was confirmed in another experiment in which ligated ovarian fragments were treated with nerve extract for 1 hour. The eggs within such fragments lacked follicles as well as germinal vesicles, and scattered freely when the ovary was torn, whereas eggs within the control (nontreated) fragments had both follicles and germinal vesicles, and tended to adhere to each other. Histological examination made on the ligated ovarian fragments immersed in sea water containing nerve extract (500 μg/ml) for 1 hour clearly confirmed these observations made on living material. As shown in Figure 2, oocytes within the treated ovarian frag-

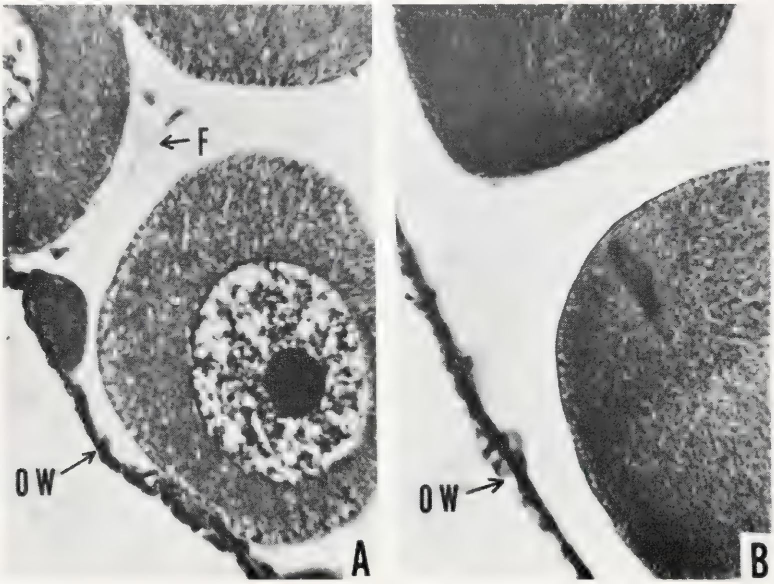

FIGURE 2. Maturation of oocytes within ovary brought about by nerve extract. (A) Section of ovarian fragment immersed for 1 hour in sea water (control), OW, ovarian wall; F, follicle. (B) Section of ovarian fragment immersed in sea water containing nerve extract for 1 hour. Note that oocytes are detached from the ovarian wall and undergoing first maturation division Follicles surrounding oocytes disappear.

ments were detached from the ovarian wall and were undergoing the first maturation division, whereas oocytes in control fragments kept in sea water alone for the same period were attached to the ovarian wall, and between them a follicular envelop was observed. Germinal vesicles of the control oocytes remained intact.

These experiments showed that although contraction of the ovarian wall seemed to be essential for expelling the eggs from the ovary, there existed a marked difference between the portions treated with nerve extract and the controls with respect to the readiness with which eggs were released.

Further, when an ovarian alveolus was isolated from an ovary and its wall slit by fine forceps in sea water, the alevolar wall contracted suddenly, discharging a mass of eggs from the slit, and turning inside-out because of the presence of eggs adhering to its inner surface and to each other (Fig. 3). Most of the eggs in isolated alveoli treated with nerve extract were freely discharged after a certain period even without a slit being made.

From these results, it is concluded that the action of the neural substance results in freeing the eggs from adherence to each other and to the inner surface of the gonadal wall, by dissolving or rupturing the follicles.

Spawning of ovarian fragments in sea water lacking divalent cations

Since bivalent cations such as calcium and magnesium are well known to stablize intercellular cementing substances, the effects of deficiency of such divalent

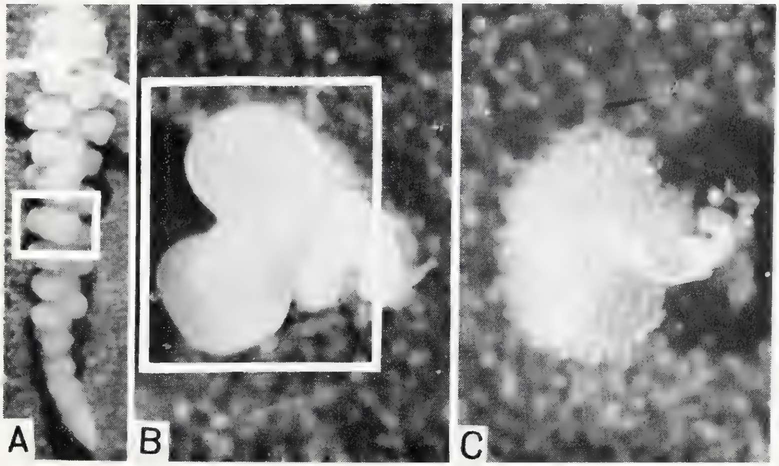

FIGURE 3. Adherence of eggs to each other and to the inner surface of the gonadal wall. (A) Ligated ovarian fragment of *Asterias amurensis,* white quadrangle shows an ovarian alveolus; (B) isolated ovarian alveolus; (C) same alveolus turns inside-out after its wall is slit. Note adherence of eggs to each other.

cations on spawning were next investigated. Isolated ovaries were transferred to magnesium-free sea water and rinsed three times with renewal of the medium, for five minutes each time. Small ovarian fragments were then removed and kept in sea water containing magnesium at various concentrations. Within half an hour the ovarian fragments immersed in media containing magnesium at lower concentrations began to shed eggs (Fig. 4). The critical concentration of magnesium at which spawning occurred was usually $3 \times 10^{-3}\,M$ and the degree of spawning increased as the concentration of magnesium decreased. The intervals preceding the discharge of eggs and amounts of discharged eggs varied according to the condition of the gonad in the breeding season in the same way as in the nerve extract experiment. Later in the breeding season the ovary was usually more sensitive to spawning inducers.

The effect of lack of calcium on spawning was next studied. Ovaries were isolated and rinsed twice with calcium-free sea water before being cut into small pieces. The fragments were further washed five times (1 minute each) with fresh calcium-free sea water; a total of about ten minutes was needed for preparation. Ovarian fragments kept in calcium-free sea water failed to discharge eggs in contrast to those treated with magnesium-free sea water. Intensive spawning, however, suddenly occurred when these fragments treated with calcium-free sea water for a prolonged period were returned to sea water. As shown in Table I, a minimum treatment with calcium-free sea water of 45 minutes was required to induce copious spawning.

When ovarian fragments treated with calcium-free sea water were transferred to sea water containing calcium at various concentrations, the discharge of eggs became more intense as the concentration of calcium ions rose. Table II shows a representative result of such experiments. The concentration of calcium required for significant spawning was above $10^{-3}\,M$ in this case.

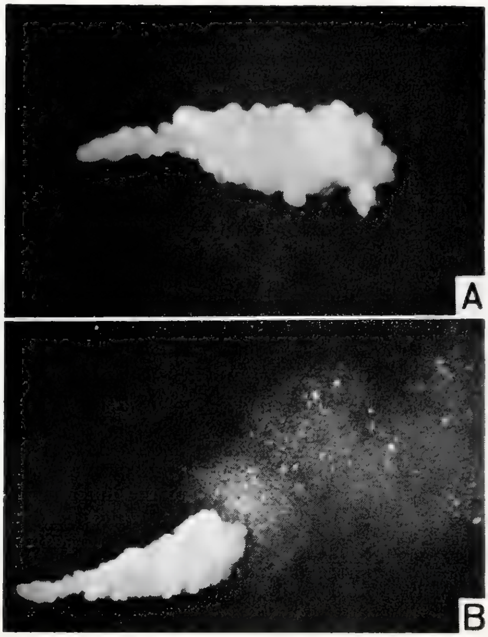

FIGURE 4. Spawning of ovarian fragment treated with magnesium-free sea water; (A) ovarian fragment before treatment with magnesium-free sea water; (B) same fragment discharging eggs in magnesium-free sea water.

In order to determine whether or not the effect of calcium deficiency is reversible, ovarian fragments, ligated to prevent discharge of eggs, were kept in calcium-free sea water for 50 minutes and then placed in sea water for appropriate periods up to 90 minutes. When cut at the ligated portion such fragments began to shed

TABLE I

Induction of spawning in ovarian fragments of Asterias amurensis *when they were kept in calcium-free sea water for various periods and then transferred to sea water*

Duration of treatment with calcium-free sea water (minutes)	Interval preceding discharge of eggs after transfer to sea water	Degree of spawning 30 minutes after transfer to sea water
10	(no spawning)	−*
15	(no spawning)	−
20	4–5 minutes	±
25	1–2 minutes	+
30	4–5 seconds	+
35	2–3 seconds	++
40	2–3 seconds	++
45	at once	+++
50	at once	+++
60	at once	+++

* −: no spawning; ±: very small amount of eggs; +: small amount of eggs; ++: intermediate amount of eggs; +++: large amount of eggs (ovarian fragments practically empty).

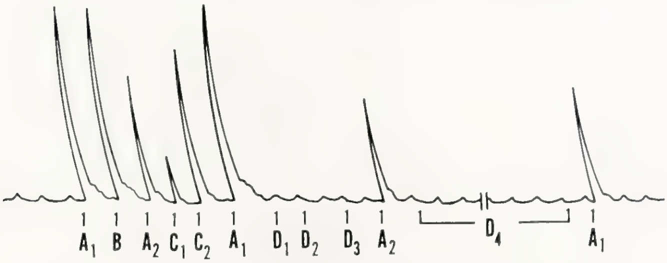

FIGURE 5. Contraction-inducing effects of crude and gel-filtrated extracts of starfish radial nerve on superfused rat uterus; tracings of original kymographic recordings: A$_1$, oxtocin (50 μU); A$_2$, oxtocin (25 μU); B, crude nerve extract of *Asterina pectinifera* (100 μg of dry nerve); C$_1$, crude nerve extract of *Asterias amurensis* (100 μg of dry nerve); C$_2$, same (200 μg of dry nerve); D$_1$, nerve extract of *Asterias amurensis* gel-filtrated on Sephadex G-25 (750 μg of dry nerve); D$_2$, same (360 μg of dry nerve); D$_3$, same (75 μg of dry nerve); D$_4$, same (750 μg/ml solution was perfused for 1 hour).

eggs at once. The degree of spawning was intense so that the fragments were practically emptied. Since the follicles were no longer observed around the eggs, this irreversibility of the effect of calcium deficiency seemed to be due to the fact that the subsequent addition of calcium failed to repair the broken follicles which had disintegrated under the previous condition of calcium lack. In the absence of calcium, ovarian fragments failed to spawn even in the presence of salt-free nerve extract (about 25 μg or 100 μg lyophilized nerve/ml), although breakdown

TABLE II

Induction of spawning in ovarian fragments of Asterias amurensis *when they were treated with calcium-free sea water and then transferred to sea water containing calcium at various concentrations*

Concentration of calcium in test sea water	Duration of treatment with calcium-free sea water (minutes)				
	15	30	45	60	90
3×10^{-2} *M*	4–5 minutes* ±**	4–5 seconds* ++**	at once* +++**	at once* +++**	at once* +++**
10^{-2} *M*	—	4–5 seconds +	at once +++	at once +++	at once +++
3×10^{-3} *M*	—	20 seconds ±	at once ++	at once +++	2–3 seconds +++
10^{-3} *M*	—	1–2 minutes ±	6-7 seconds ++	6–7 seconds ++	2–3 seconds +++
3×10^{-4} *M*	—	—	3–4 minutes +	20 seconds +	20 seconds +
10^{-4} *M*	—	—	—	—	—

* Interval preceding discharge of eggs after transfer to medium containing calcium.
** Degree of spawning; see Table I.

of the germinal vesicles occurred within the fragments after 30 minutes. These fragments began to spawn immediately after addition of calcium.

Relation between contraction of ovary and nerve extract

It is evident that the contraction of the ovarian wall is essential for starfish spawning, in order to expel the eggs from the ovary. This contraction of the ovarian wall seems to be due to its muscular component. Although muscle cells were histologically difficult to recognize in the wall of the distended ovary during the breeding season, contracting muscle cells were clearly observed in histological preparations of the wall of spent ovarian fragments after treatment with nerve extract.

Some experiments were conducted to investigate (1) the effect of known contraction-inducing agents such as potassium chloride and acetylcholine chloride on the spawning of *Asterias amurensis;* (2) the contraction-inducing ability of nerve extract as checked by the superfusion method using rat uterus, a common pharmacological test, and (3) the effect of contraction-inducing agents and nerve extract on the contraction of the isolated gonadal wall of starfish.

When 5 ml of 5/9 *M* potassium chloride were injected into the coelomic cavities of *Asterias* (about 300 g in body weight), small amounts of sperm or eggs were discharged from the gonopores within a few minutes in some starfish. However, this release of gametes soon ceased. Injection of 5 ml of sea water containing 5×10^{-3} *M* acetylcholine chloride had the same effect. Addition of eserine at low concentrations to acetylcholine solution did not alter the results.

In vitro experiments, in which either 10 ml of sea water containing 5 ml of 5/9 *M* potassium chloride, or acetylcholine chloride in sea water (10^{-3} *M* final concentration) were used, showed that a small amount of eggs was immediately discharged from the cut surface, but the discharge soon ceased. When electric stimulation (20–40 volts, alternating current, for 10 seconds) was applied to a small ovarian fragment in sea water by placing it between two electrodes 2 cm apart, the reaction of the fragment was similar to that seen on treatment with potassium chloride or acetylcholine chloride. Under a dissection microscope, the eggs within the ovary were observed to elongate along the long axis of the ovarian alveoli when treated with these contraction-inducing agents suggesting that the ovarian wall was under tension. On the other hand, ovarian fragments taken from the same female and treated with nerve extract spawned copiously until they were practically empty.

It should be noted that the induction of this small-scale spawning by contraction-inducing agents such as potassium chloride and acetylcholine depended much upon the state of gonads. Some ovarian fragments, whose sister fragments spawned heavily with nerve extract, failed to respond to such agents, while others discharged eggs to a certain degree. Testis fragments usually responded well to these treatments and shed a cylindrical mass of sperm from the cut surface, suggesting that the gametes could be expelled more readily from the testis than from the ovary.

The effect of starfish nerve extract on muscle tissue was examined with superfused rat uterine muscle isolated from a proestrus rat (Gaddum, 1953), using synthetic oxytocin as the reference standard. Representative kymographic recordings show the contractability of the uterine preparation induced by oxytocin (Fig. 5A), and by crude nerve extracts of *Asterina pectinifera* (Fig. 5B) and *Asterias amurensis* (Fig. 5C). However when these crude extracts were gel-filtrated on a Sephadex G-25 column in order to remove possible contaminating substances of small molecular weight, the contraction-inducing ability was lost; contraction was no longer observed with various dosages such as 0.1 ml of 750 to 7,500 μg of lyophilized nerve of *Asterias* per ml. (Fig. $5D_{1-3}$). Finally, no contraction of the uterus was observed (Fig. $5D_4$), when 50 ml of the gel-filtrated nerve extract (750 μg lyophilized nerve per ml) was superfused for 1 hour, although this extract was very effective in inducing spawning, causing isolated ovaries to begin shedding their eggs within 20 minutes. The same uterine preparation contracted strongly on addition of 0.1 ml of 50 μU oxytocin even after these prolonged experiments (Fig. 5, last A peak). It is therefore concluded that the neural substance responsibile for spawning by itself was not the agent which caused contraction of the rat uterine muscle. The contraction observed following treatment with the crude nerve extract seems to be ascribable to some factor(s), contained in the nerve extract, other than the so-called shedding substance.

In order to compare the effffect of potassium chloride and acetylcholine chloride (Kanatani, 1967) on the contraction of the ovarian wall with that of the shedding substance, some experiments were conducted using ovary of *Asterias*. Kymographic recordings showed that strong contraction of isolated ovarian wall preparations was induced by both potassium chloride and acetylcholine chloride, and that the capacity of the gel-filtrated nerve extract to cause contraction of the ovarian wall was, if any, very slight, suggesting that the shedding substance acts on the ovary to induce spawning by some way other than by causing the ovary to contract.

Effect of treatment with hyaluronidase and trypsin on spawning

Some experiments were conducted to test whether treatment with hyaluronidase or trypsin would cause induction of spawning.

Injecting either 0.5 ml of sea water containing 0.5% hyaluronidase (NBC, 300 U/mg) or 0.2 ml of sea water containing 0.1% trypsin (Sigma, 10,000 U/mg) into the isolated ovarian fragments (*ca.* 3 cm in length) of *Asterias amurensis* failed to induce discharge of eggs. Ovarian fragments immersed in these solutions also failed to spawn. Masses of eggs adhering to the cut surfaces did not show any sign of dispersion. These results suggest that the intercellular cementing substance between oocytes resists the action of these enzymes.

Effect of nerve extract on spawning when applied from outside of the body

The neural substance was found in the coelomic fluid only when the starfish were undergoing natural spawning (Kanatani and Ohguri, 1966). Some experiments were carried out with *Asterias amurensis* to determine whether or not the active substance is released from the radial nerves *into the surrounding sea water* and then taken by the same or a neighboring animal to stimulate the release of gametes, as postulated by Chaet (1966b; 1967, pp. 16–17).

A total of sixty starfish (50–110 g in body weight) were kept separately for 4–8 hours in 500 ml of sea water containing nerve extract in various concentra-

TABLE III

Effect of sea water containing nerve extract on spawning when applied from outside of the body in Asterias amurensis

Date of experiment	Concentration of nerve extract	Period of treatment (hours)	No. of animals used	No. of animals spawned	No. of animals* spawned after injection with nerve extract
March 24	200 μg/ml	4	10 ♂7 ♀3	0	8** ♂5 ♀3
	100 μg/ml	4	10 ♂3 ♀7	0	10 ♂3 ♀7
	0 (control)	4	10 ♂3 ♀7	0	10 ♂3 ♀7
April 21	50 μg/ml	4.5	10 ♂1 ♀8 ?1	0	5** ♂1 ♀4
	25 μg/ml	4.5	10 ♂3 ♀7	0	6** ♂2 ♀4
April 23	200 μg/ml	8	10 ♂5 ♀5	1	9*** ♂5 ♀4
	100 μg/ml	8	10 ♂4 ♀6	1	9*** ♂4 ♀5
	0 (control)	8	15 ♂6 ♀9	2 ♂1 ♀1	13*** ♂5 ♀8

* After treatment with the test solution, 1 ml of nerve extract (20–100 μg/ml) was injected into the coelomic cavity.

** Gonads poorly developed, or had already spawned, in animals which did not spawn on injection with nerve extract.

*** No injection was performed with animals which had already discharged their gametes.

tions (25–200 μg of lyophilized nerve per ml). Another twenty-five starfish kept in sea water alone served as controls. During the 8-hour treatment, the sea water was aerated throughout the experiment. After such treatment 1 or 2 ml of the nerve extract (20–100 μg/ml; the highest dosage was 100 μg/animal) was injected into the coelomic cavity in order to check the spawning capacity. The results are shown in Table III. Forty animals, both males and females, kept in sea water containing nerve extract (25–200 μg/ml) for 4 to 4.5 hours on March 24 and April 21 failed to spawn. However, when these animals were washed, transferred to fresh sea water, and injected with the nerve extract, they discharged eggs or sperm within 1 hour. Animals which did not spawn after injection were found on autopsy to have very small gonads. Although two of the 20 animals kept in sea water containing nerve extract (100–200 μg of dry nerve per ml) for 8 hours on April 23 spawned, two of the 15 control animals also discharged gametes. On the same day, natural spawning was observed in several starfish kept in other aquaria, suggesting that spawning in this case was not due to the action of the applied nerve extract. Both males and females which had failed to spawn in sea water containing nerve extract discharged sperm or eggs within 1 hour after injection of nerve extract (100 μg of dry nerve per animal).

In the final experiment of this series, the arms of starfish were slit with scissors for about 2 cm along the ambulacral plate, in order to introduce the external medium into the arm. In these experiments, six (3 males and 3 females) of the 7 starfish kept in sea water containing nerve extract (100 μg/ml) discharged their gametes within 2 hours. Further, four (2 males and 2 females) of the six exposed to 50 μg/ml and four (2 males and 2 females) of the five exposed to 25 μg/ml discharged their gametes.

From these experiments it is clear that the neural substance responsible for spawning has no effect when it is applied from the outside of the starfish body.

Discussion

Starfish spawning, as induced by injecting the nerve extract into the coelomic cavity or by treating isolated ovarian fragments with sea water containing nerve extract, is explained as follows. The polypeptide hormone contained in the radial nerve (Kanatani and Noumura, 1962; Kanatani, 1967, 1969; Chaet, 1967) is liberated so that it reaches the coelomic cavity, and there directly acts on the ovary from its surface to bring about dissolution of an intercellular cementing substance between the follicle cells and the ovarian wall, making the eggs within the ovary freely movable. Under these conditions the ovarian wall, whose contractability seems to be ascribable to its muscular component (Chaet, 1966a; Kanatani, 1967; Schuetz and Biggers, 1968), can contract actively and force out the individual eggs, thus bringing about spawning. The active substance itself does not seem to be a direct cause of the contraction of the ovarian wall, as will be discussed below.

A contrary opinion has been expressed by Chaet (1966ab), who believes that this substance causes the ovarian wall to contract actively, thus expelling the eggs. If the nerve extract brings about spawning by simply causing muscular contraction, spawning should also be induced by treatments which cause contraction of the gonadal wall. However, as shown in the present study, potassium chloride, acetylcholine chloride and electric stimulus all fail to induce significant spawning. In the case of sea urchins, application of these contraction-inducing agents is very successful in inducing spawning (Iida, 1942; Iwata, 1950; Harvey, 1952, 1953; Iwata and Fukase, 1964ab). Since the histological constituents of the ovarian walls in sea urchins and starfish are almost identical (Hamann, 1885; Kawaguti, 1965; Kanatani, unpublished data), and since the isolated ovarian wall of starfish itself contracts vigorously on treatment with potassium chloride and acetylcholine (Kanatani, 1967), the failure of contraction-inducing agents to induce spawning in starfish should be ascribed to some other cause. Observation of isolated ovarian alveoli in the present study suggests that the masses of eggs adhering to each other or to the inner surface of the gonadal wall obstruct the release of individual eggs, so that they resist the contraction of the ovarian wall. Sea urchin oocytes, on the other hand, mature within the ovary and are already free from the follicles long before spawning takes place (Fuji, 1960). This condition of the ovary seems to explain why the spawning of sea urchins can be readily induced by some contraction-inducing agents.

Experiments to test the contraction-inducing activity of starfish nerve extract, with rat uterus as well as with starfish ovarian wall, revealed that crude nerve extract has some effect in causing muscle tissue to contract. However, when the nerve extract was partially purified by gel-filtration in order to remove possible contraction-inducing contaminants such as acetylcholine (Unger, 1962), it no longer showed such activity. It is therefore concluded that the ability of the active peptide to cause contraction of the ovarian wall is, if present at all, very slight, suggesting that its primary and main action is to dissolve the cementing substance surrounding the eggs. It is to be noted that our previous results (Kanatani, 1964), obtained in an experiment measuring changes in pressure within the ovarian fragments, should be re-examined since the experiment used crude nerve extracts without gel-filtration, and it is possible that contraction-inducing agents contaminating the extracts may affect pressure change.

Chaet's observation (1966a, p. 50) that the ovary began to contract in the presence of nerve extract several minutes before eggs were extruded through the gonopore (about 25 minutes after the treatment began) can be explained in another way; during this period of treatment the extract acts on the cementing substance within the ovary, to breakdown the follicles and to set free the eggs, so that individual eggs can be displaced within the distended ovary (whose wall is under considerable tension). As a result of this change, the surface area of the ovarian wall decreases, causing the ovary to decrease in length. A pronounced decrease in the length of ovary is seen after the oocytes have been discharged. Therefore, decrease in ovarian length after treatment with sea water containing varying concentrations of calcium and a constant amount of gel-filtrated nerve extract (Schuetz and Biggers, 1968) does not seem to contradict the present notion. Furthermore, in Chaet's experiment (1966a), the question arises as to why such a long period as 25 minutes elapses between the onset of treatment and the commencement of contraction. Since contraction of the ovarian wall does not require such a long latent period, this period is thought to represent the time needed for breakdown of follicles in the ovary. In our experience, potassium chloride or acetylcholine causes immediate contraction of the ovarian wall (Kanatani, 1967).

Although there is no doubt that contraction of the ovarian wall is a direct cause of the release of individual oocytes which have been liberated from binding to each other and to the gonadal wall, as a result of the breakdown of follicles under the influence of hormonal peptide, there is as yet no direct evidence available to explain how this contraction is realized. Although simple tearing of the ovarian alveolus causes the strong contraction of its wall, the effect of mechanical stimulation is not excluded in this case. That the isolated hormone peptide can induce spawning of ovarian fragments (Kanatani, unpublished data) seems to exclude the possibility that some unknown contraction-inducing substance, which might be released simultaneously from the nerve at the time of release of the hormonal peptide, is required to bring about spawning. What directly causes the active contraction of the ovarian wall remains unsolved; induction of spawning by keeping the ovarian fragments in magnesium-free sea water or by transferring them from calcium-free sea water to normal sea water (Kanatani, 1964, 1967; Schuetz and Biggers, 1968) suggests that no specific contraction-inducing agent is required.

The occurrence of spawning in ovarian fragments immersed in magnesium-free sea water without nerve extract, or in sea water lacking both calcium and magnesium without nerve extract (Kanatani, 1964) suggests, besides the dissolution of cementing substance, that magnesium may inhibit the discharge of oocytes;

this is in accord with the statement of Schuetz and Biggers (1968). According to Iwata and Fukase (1964b) magnesium anaesthetizes the neuro-muscular junction, and application of excess magnesium inhibits the spawning of sea urchins induced by acetylcholine. In calcium-free sea water without nerve extract ovarian fragments do not discharge their eggs. In this case, the presence of magnesium ions may be involved in the failure of spawning. The induction of spawning after adding calcium to calcium-free sea water might be a problem of the ionic balance between calcium and magnesium. On the other hand, although calcium ions are believed to play an important role in starfish spawning (Mecklenburg and Chaet, 1964; Kanatani, 1967; Schuetz and Biggers, 1968) ovarian fragments undergo discharge of eggs when they are kept in sea water lacking both calcium and magnesium ions (Kanatani, 1964; 1967). Some technical difficulty, always encountered in experiments using isolated whole ovary or fragments to remove the target ions exclusively, makes it impossible to draw any definite conclusion as to the effect of a given ion. At present, the exact effect of various ions on starfish spawning is far from clear. We can only say that the absence of divalent cations in sea water causes the breakdown of follicles and this is a prerequisite for such artificial spawning.

With respect to the route by which the active substance travels from the radial nerve to the gonad, Chaet (1966b, p. 268) has suggested that "the substance is released from the oral surface of the radial nerve into the sea water, and if not diluted below a threshold level, stimulates the release of gametes from its neighbor." At the same time, "a threshold level of shedding substance would be transported into the coelmic cavity of the original starfish to stimulate its gonad." Although this opinion, that the substance may act as a pheromone rather than hormone, is of interest, the data obtained from the experiment which tested the effect of nerve extract applied from the outside of the body show that the substance is unable to enter the coelomic cavity. In these experiments, one of the doses of nerve extract used (500 ml of sea water containing 200 μg/ml of nerve extract) corresponds to the quantity which is contained in the radial nerves of a single starfish. Since introduction of sea water containing nerve extract at various concentrations into the coelomic cavity through the incision made along the ambulacural plate brought about spawning in almost all the starfish treated, there is no question about the concentration of the nerve extract used. Therefore the phenomenon often observed in the laboratory, that several starfish in the same aquarium begin to shed simultaneously, should not be explained as stimulation of spawning by shedding substances secreted by other starfish. There seem to be some environmental factors, unknown external stimuli, which directly or indirectly induce the release of the active substance from the nerves into the coelomic cavity within each individual.

In regard to the mode of action of the active peptide on induction of spawning and oocyte maturation, recently an intermediate substance was discovered (Kanatani and Shirai, 1967; Schuetz and Biggers, 1967). This second active substance is produced in the ovarian wall under the influence of the active peptide and brings about oocyte maturation as well as spawning. Detailed data as to the production of this substance and its chemical nature will be described in the next paper of this series.

We wish to express our gratitude to Dr. T. Hirano for measuring the contraction of rat uterus, to Dr. J. C. Dan for reading the manuscript, and to Misses H. Ito and K. Sawayama for their technical assistance. Our thanks are due to the directors and the staff of the Marine Biological Stations at Misaki and Akkeshi for putting the research facilities of the stations at our disposal.

SUMMARY

1. When a water extract of radial nerves of the starfish, *Asterias amurensis,* was locally applied to one part of an isolated whole ovary for an appropriate period and then small slits were made on the ovarian wall, intense discharge of eggs occurred only in the treated portion.

2. Eggs within the nontreated part of the ovary were observed to adhere to each other and to the gonadal wall by means of the follicle layer surrounding them.

3. Eggs within ligated ovarian fragments which had been treated with nerve extract lost their follicles and underwent maturation. These eggs were found to be freely movable.

4. Artificial spawning could be induced without using nerve extract; ovarian fragments immersed in Mg-free sea water released their eggs after a certain interval, while those treated with Ca-free sea water for an appropriate period spawn after subsequent addition of calcium.

5. Breakdown of follicles occurred within an ovary treated with calcium-free sea water.

6. Treatment with contraction-inducing agents such as potassium chloride and acetylcholine did not bring about spawning, although they caused contraction of isolated ovarian wall preparations.

7. Although the crude nerve extract caused muscular contraction in an isolated rat uterus, gel-filtrated nerve extract failed to induce contraction of either rat uterus or starfish ovarian wall.

8. These findings suggest that the action of the neural substance which is responsible for spawning acts within the ovary to induce breakdown of the follicles surrounding the oocytes.

9. Treatment with hyaluronidase and trypsin failed to induce spawning, suggesting that the intercellular cementing substance between the oocytes resists the action of these enzymes.

10. The neural substance failed to induce spawning when it was applied from the outside of the body.

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REGENERATION OF COMPLETE HYDRA FROM ISOLATED EPIDERMAL EXPLANTS [1]

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A number of workers have studied the regenerative properties of isolated cell layers from coelenterates. Gilchrist (1937) and Steinberg (1963) reported the ability of isolated epidermis from *Aurelia* scyphistomae to regenerate into complete polyps. Zwilling (1963) found that isolated pieces of epidermis in *Cordylophora* regenerated into complete polyps but that isolated gastrodermis disintegrated. Although Papenfuss and Bokenham (1939) reported that neither cell layer in *Hydra* would regenerate, later work has shown that the gastrodermis of both brown and green hydra can regenerate whole animals. Normandin (1960) reported complete polyp regeneration from gastrodermal explants of *Hydra oligactis,* and Haynes and Burnett (1963) obtained similar regeneration from isolated gastrodermis of *Hydra viridis.*

However, regeneration of complete hydras both from isolated epidermis and from isolated gastrodermis of the same species has not been reported previously. The present paper reports the development of gastrodermis, and finally whole animals, from epidermal explants of *Hydra oligactis* and *Hydra pseudoligactis,* demonstrating the totipotency of each cell layer in *Hydra.*

The individual cell layers must be separated in order to test their regenerative potential. Various methods have been used to do this. Early workers relied on mechanical or surgical techniques, scraping or cutting away the gastrodermis. In *Aurelia* scyphistomae manual dissection is effective because a thick layer of mesoglea separates the cell layers. Papenfuss (1934) obtained endoderm-free cylinders of epidermis by scraping away gastrodermal cells from everted hydra, a laborious process. More recently, chemical or combination chemical and mechanical methods have been employed. Normandin (1960) removed the hypostome and tentacles and treated the body column with pancreatin and amylase to digest the mesoglea. Haynes and Burnett (1963) used a trypsin solution. Also in Burnett's laboratory Muscatine's solution (1961) brought to pH 2.5 with HCl has been used to remove the epidermis (Lenhoff and Muscatine, 1963). The acid and enzyme treatments, however, invariably damage the epidermal cells and no epidermal explants have shown any regenerative ability after such treatment.

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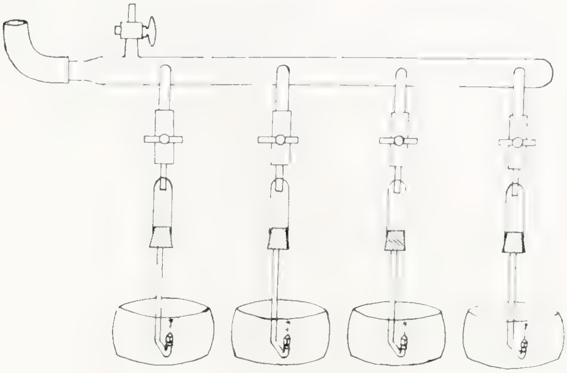


FIGURE 1. Perfusion apparatus (see text for explanation).

The present study of epidermal regeneration uses a simple perfusion technique which separates the cell layers cleanly without apparent injury to either epidermis or gastrodermis.

METHODS

Separation of epidermis and gastrodermis is accomplished by perfusing the gastrovascular cavity with Haynes' solution (0.2% NaCl, 0.2% $CaCl_2$, 0.03% $MgSO_4$, and 0.01% $KHPO_4$ made up in glass distilled water).

The perfusion fluid is contained in a reservoir supplying a glass manifold to which the individual perfusion units are attached (Fig. 1). Each unit has an

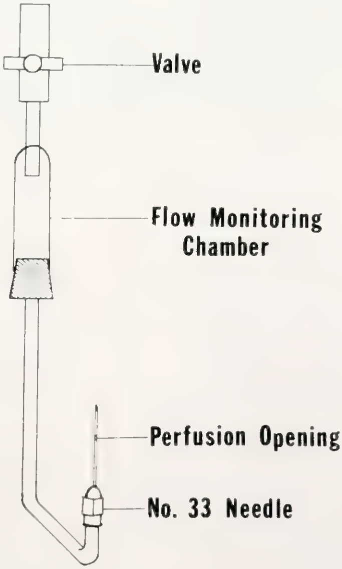


FIGURE 2. Perfusion unit.

adjustable clamp for admitting the perfusate, a transparent chamber for monitoring flow rate, and a J-shaped piece of glass tubing which carries a specially perforated #33 hypodermic needle on its lower end (Fig. 2). A hole is made in the side of the needle with a fine grinding wheel about $\frac{1}{3}$ inch from the tip and the tip sealed with epoxy (Hysol 1C White).

The hydra is threaded on the needle with the point entering the pore in the basal disc and coming out the mouth and the animal positioned so that the opening lies in the gastrovascular cavity. Since the hydra often works its way off the needle it is maintained in position by forcing a small piece of Parafilm over the needle tip (Figs. 3 and 4). The needle together with the lower part of the perfusion unit is immersed in a dish of standard culture medium (Loomis and Lenhoff, 1956) made up with distilled water. It is not necessary to maintain a specific rate of flow; the purpose of the monitoring chamber is to insure that flow does not stop from contraction of the animal around the needle.

The temperature of the perfusion dishes was maintained between 10–15° C throughout the separation process.

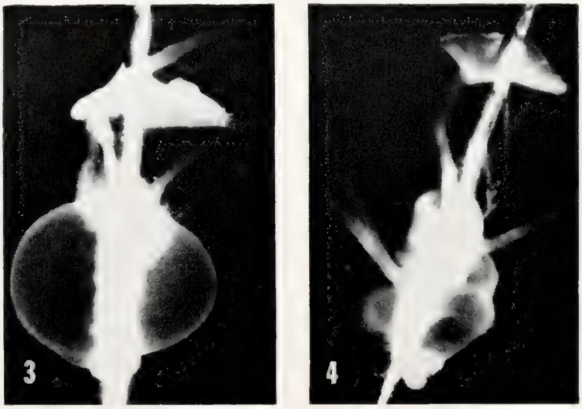


FIGURE 3. Hydra on perfusion needle showing epidermal separation. × 66.
FIGURE 4. Hydra on perfusion needle showing spontaneous eversion. × 66.

Separation is usually evident without the use of a microscope. Following separation the lower part of the perfusion unit, still immersed in its dish of culture medium and with the hydra still on the needle, is detached from the monitoring chamber and mounted in a clamp under a dissecting microscope. Iridectomy scissors and No. 5 watchmaker forceps are used for removing the epidermis. Once punctured, the separated epidermis tends to collapse inward, but by grasping the cut edge with the forceps it can be lifted away from the gastrodermis. Then by exerting gentle tension the epidermis can be cut away, the animal meantime revolving on the needle. In this way it is possible to obtain large pieces or cylinders of epidermis completely free from gastrodermis.

This technique for cell layer separation was tested on three species of brown *Hydra*: *H. fusca* (8 animals), *H. oligactis* (31 animals), and *H. pseudoligactis* (458 animals). It was also tested on *Hydra viridis* (18 animals). The brown

Hydra were taken from stock cultures maintained by the method of Loomis and Lenhoff (1956) with distilled water substituted for tap water, and the green *Hydra* were cultured by the same method but using commercially supplied spring water instead of tap water.

Regeneration studies were made on *H. fusca* (3 animals), *H. oligactis* (4 animals), and *H. pseudoligactis* (91 animals). Epidermal explants were cultured individually in small (6 cm) petri dishes in approximately 20 ml of standard culture medium (Loomis and Lenhoff, 1956), in standard culture medium containing 2½–3% Haynes' solution, and in two cases standard medium and Haynes' solution in equal proportions. Most of the cultures were kept at room temperature (20°–24° C), though a few were kept at 12° C.

Animals selected for histological study were fixed in Bouin's or glutaraldehyde, stained in toluidine blue at pH 8, dehydrated in alcohol, and mounted in permount.

RESULTS

Cell layer separation

Separation of epidermis from gastrodermis by perfusion was successful in all species of *Hydra* tested. Frequency of separation was analyzed by species, temperature, nutrition and season. In the four species tested under a variety of conditions of nutrition, budding, and temperature, the separation frequencies (including all degrees of separation) were as follows: *H. fusca*, four separations out of eight animals tested; *H. oligactis*, eight separations out of 31 animals tested; *H. pseudoligactis*, 218 separations out of 458 animals tested; *H. viridis*, four separations out of eighteen animals tested.

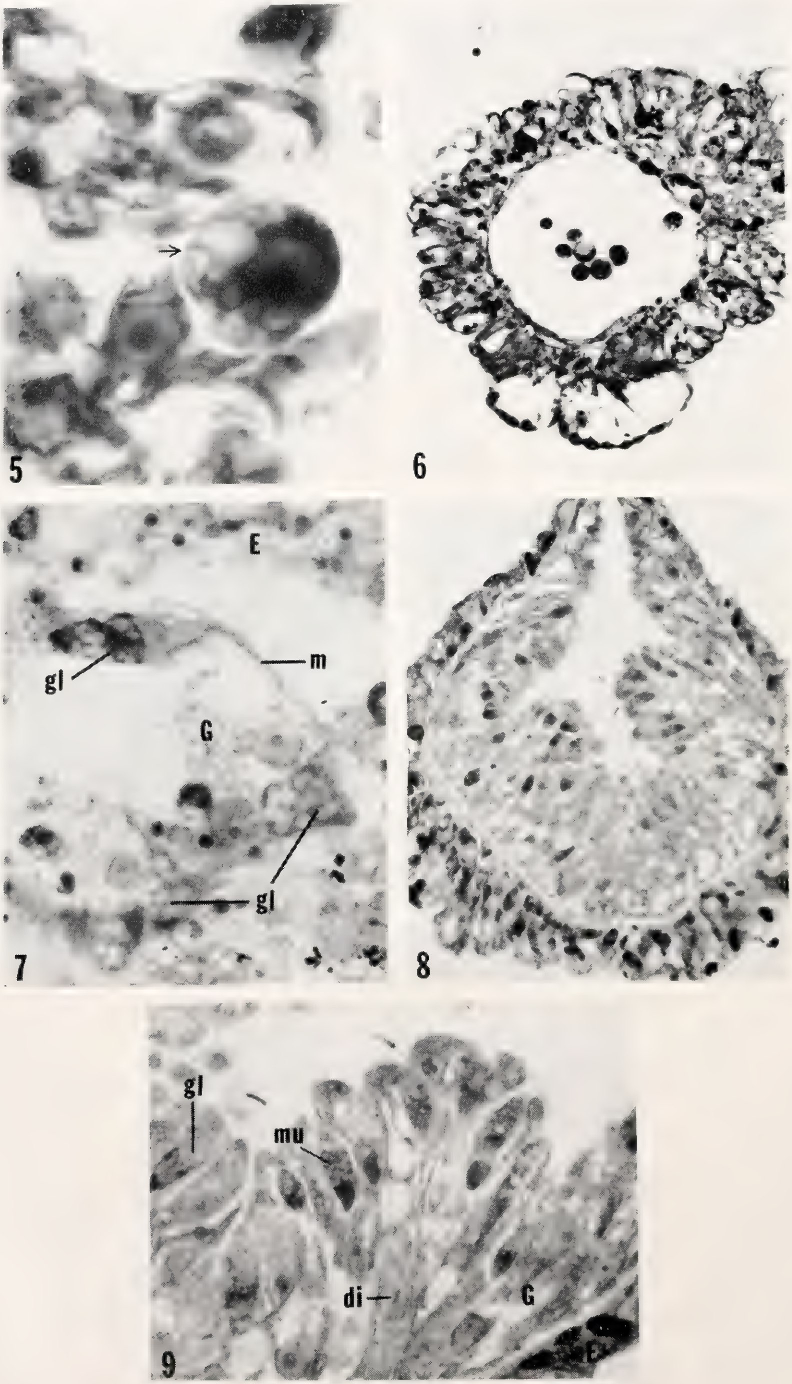
The effect of temperature on separation was tested with *H. pseudoligactis*. In a range from 5°–30° C the frequency of separation is inversely related to temperature. Between 25°–30° C one out of sixteen animals separated (6.25%). Between 15°–25° C 36 out of 100 animals separated (36%). Between 5°–15° C 173 out of 344 animals separated (50%). These figures include all degrees of separation.

Separation frequency also changed seasonally. From late October through March, when this work was done, the frequency of separation increased through November and December to a maximum in January and February, then declined again in March.

An additional factor correlated with separation frequency was nutrition. An analysis of this for *H. pseudoligactis* showed a separation frequency of 36% when perfusion occurred within 44 hours of feeding (177 animals), and 52% when perfusion occurred 45–100 hours after feeding (293 animals).

When the *Hydra* first accommodates itself to impalement the mouth usually closes around the needle so when perfusion begins the animal swells up like a miniature balloon. However, the mouth soon opens to establish an equilbrium.

Separation may occur at any time from five minutes up to two hours, with the majority of separations taking place within the first half hour. Separation may begin at any point between the tentacle ring and the basal disc. It proceeds as a wave proximally or distally or both from the point of origin, and also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Figures 5–9.

circumferentially often going completely around the animal. The result is the formation of a fluid-filled space between the inner and outer cell layers.

The locus of separation is in the mesoglea and lies closer to the gastrodermis than the epidermis. In stained sections of whole animals in which separation has occurred most of the mesoglea is seen attached to the epidermis. Separation of the mesoglea from gastrodermis can also be seen in the effect of continued perfusion of separated animals. After three to six hours of continued perfusion of a separated animal, tears occur in the epidermis exposing blisters of mesoglea. When this happens in a maximally separated animal the tears encircle the animal and the epidermis retracts or sloughs away, leaving behind a fluid-filled sac of naked mesoglea.

The extent of separation varies from a small blister of epidermis to maximum separation in which the entire epidermis between tentacles and basal disc forms a nearly transparent sphere in which the gastrodermis appears as a spindle contracted around the needle (Fig. 3). Ordinarily separation does not occur in the hypostome or tentacles or at the basal disc, and frequently only partial separation occurs in the peduncle. There is no separation around the projecting portion of a developing bud.

Occasionally a maximally separated animal will begin to evert on the needle. When this occurs the outrolling lip of the hypostome carries the tentacle ring and separated epidermis basally over the gastrodermis (Fig. 4), often detaching the epidermis of the basal disc from the gastrodermis. In such cases it is possible to cut below the tentacles and remove virtually the entire epidermis from the animal.

When a piece of epidermis is removed it curls up, scroll-like, along its proximo-distal axis, presumably due to contraction of the muscle fibers at the base of the epithelio-muscular cells. When an epidermal cylinder is removed it contracts to form a doughnut-like ring.

Regeneration of epidermal explants

A total of 98 epidermal explants were tested for regenerative ability. These differed in size and original location on the body column. Small explants, $\frac{1}{8}$ or less of the body column epidermis, invariably disintegrated within 12–48 hours. Larger pieces, up to entire epidermal cylinders, showed varying degrees of regeneration of a coelenteron and an inner gastrodermal cell layer in 17% of cultures, though regeneration was not a one-to-one function of the size of the explant. Frequency and degree of regeneration did not increase after the explant exceeded approximately one-half the total body column epidermis.

FIGURE 5. Stage I regenerate, *H. fusca*; note zymogen-like cell among epidermal cells (arrow), Toluidine blue, pH 8. × 2230.

FIGURE 6. Stage II regenerate, *H. pseudoligactis*; note flattened layer of gastrodermal cells lining coelenteron, Toluidin blue, pH 8. × 890.

FIGURE 7. Stage II regenerate, *H. pseudoligactis;* E, epidermis; G, gastrodermis; m, mesoglea; gl, gland cell; Toluidine blue, pH 8. × 910.

FIGURE 8. Stage III regenerate, *H. pseudoligactis*; note fully differentiated gastrodermis, Toluidine blue, pH 8. × 360.

FIGURE 9. Stage III regenerate, *H. pseudoligactis,* E, epidermis; G, gastrodermis; gl gland cell; mu, mucous cell; di, digestive cell; Toluidine blue, pH 8. × 910.

Because of the cleanness of separation between the cell layers and the efficiency with which the epidermis could be cut away, the problem of gastrodermal contamination of epidermal explants was eliminated. With practice it was possible to secure pieces of epidermis which were completely gastrodermis-free. As a check 10 epidermal pieces were fixed, sectioned, and stained immediately after removal. In no case were gastrodermal cells present.

Seventeen of the 98 explants observed showed varying degrees of regeneration. Each of the three species tested (*H. oligactis, H. pseudoligactis, H. fusca*) showed regenerative ability.

When regeneration occurs at room temperature visible changes appear in the first 24 hours. Morphogenesis continues actively during the second day, after which the rate of change declines over the next four or five days. For convenience in reference the events in regeneration have been grouped in stages as follows:

Stage I Cavitation Depending on the size of the explant, one or more small cavities become visible with a dissecting microscope within six to twenty hours. Examination of sectioned material fixed at six and twelve hours indicates that these cavities arise through the coalescence of intercellular spaces appearing in the first few hours. At the end of this stage there is no gastrodermal layer, although the cavities may show partial bordering by a mesoglea-like membrane, and scattered cells may be present inside the membrane as well as lying free within the cavity. Zymogen-like cells may be found occasionally near the cavity or along the recesses ramifying from it, (Fig. 5). During this stage the regenerate begins to show contractile movements.

Stage II Body column, hypostome, and tentacle formation Further morphogenesis depends to some extent on the body region from which the epidermis was taken. When the explant comes from the sub-hypostome and gastric regions (most commonly), a hypostome and/or tentacle buds are the next structures to appear. When the explant includes basal disc epidermis, the regenerate may form a basal disc instead of a hypostome. In three such cases no hypostome or tentacles formed at all, even though maintained in culture for two weeks. Concomitantly there is a lengthening of the regenerate as a body column is formed. During this period extension and fusion of the previous cavities occur forming a coelenteron. The mesogleal layer is completed between the epidermis and a developing gastrodermis (Fig. 6). Early gastrodermal cells appear on the inner surface of the mesoglea as a lining of flattened epithelium (Fig. 6), which later becomes thickened and contains presumptive gland and digestive cells (Fig. 7). In this stage there is also a noticeable increase in rate and extent of spontaneous contractions.

Stage III Mouth and basal disc formation With the appearance of a mouth the regenerate becomes a potentially independent organism. Two explants were followed through to this stage though both were too small to ingest *Artemia*. A basal disc developed in only one of these, one which contained a small amount of basal disc epidermis originally. Had the other regenerate been able to feed the development of a basal disc would presumably have occurred. Histologically these regenerates show completely organized epidermal and gastrodermal layers (Fig. 8), in which the gastrodermis contains fully differentiated gland, mucous, and digestive cells (Fig. 9).

DISCUSSION

The epidermal cell type or types which give rise to the gastrodermal layer are not known. Nevertheless we believe it probable that interstitial cells play a prominent role in the process. Gastrodermal regeneration in *Hydra* may be analogous to gastrodermal regeneration in *Cordylophora* which Diehl (1969) has reported. He has followed regenerative stages in epidermal explants from *Cordylophora* in which interstitial cells move to the base of the epithelio-muscular cells soon after the epidermis is isolated. These cells become elongate, vacuolated, and differentiate directly into normal digestive cells. Paul Rose (personal communication) in our laboratory, also working with *Cordylophora*, has combined epidermis labeled with tritiated thymidine with the gastrodermis from a "cold" animal and studied the regeneration of new feeding hydranths at different time periods. He finds basophilic and secretory cells in the gastrodermis labeled, strongly implying that the labeled epidermal interstitial cells migrate to the interior of the regenerating explant and differentiate into the zymogen and mucous secretory cells.

Although our studies are in their early stages, we suspect that gastrodermal regeneration in *Hydra* will parallel that of *Cordylophora*. Different stages in the regenerative process are now being prepared for study with the electron microscope to determine ultrastructural changes in interstitial cells during the differentiation process.

Perhaps the major significance of the present study is that in *Hydra* each layer alone can give rise to the other during regeneration. Details of the regeneration of epidermis from gastrodermis have been worked out by Davis, Burnett, Haynes, and Mumaw (1966). The fact that these complementary regenerations occur in media of different ionic concentrations strengthens our belief that ionic environment is an important factor in the control of cell differentiation *in vivo* (Burnett, 1966; Macklin and Burnett, 1966).

As a variation of the epidermal regeneration described above, we have taken rings of isolated epidermis and threaded columns of isolated gastrodermis through them. In this way we have produced "composite" hydra which developed to mature feeding and budding individuals. In some cases the combinations were conspecific and in others interspecific, both types yielding successful regenerates.

SUMMARY

1. A method of separating cell layers in *Hydra* by perfusion is presented. Under optimum conditions about 50% of perfused animals show separation of epidermis from gastrodermis. With this technique it is possible to secure large pieces and even whole cylinders of living epidermis or gastrodermis free from contamination by the other cell layer.

2. Isolated explants of epidermis were tested for regeneration in *H. fusca*, *H. oligactis*, and *H. pseudoligactis*. Each species showed some regenerative ability, and complete minature hydras were obtained in *H. oligactis* and *H. pseudoligactis*. Other work (Haynes and Burnett, 1963; Normandin, 1960) has shown that isolated gastrodermis can regenerate complete hydras in *H. viridis* and *H. oligactis*. Thus the present work, showing that isolated epidermis can also regenerate complete polyps, makes *Hydra* the first animal in which it has been demonstrated that each layer alone can give rise to the other during regeneration.

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Reference: *Biol. Bull.*, **137**: 321–331. (October, 1969)

HORMONAL CONTROL OF IMAGINAL DISC REGENERATION IN *GALLERIA MELLONELLA* (LEPIDOPTERA)

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A variety of experiments have demonstrated that regeneration delays molting in insects. A penetrating study of this matter was undertaken by O'Farrell and Stock (1953) who investigated the regeneration of metathoracic leg and its effect on molting in larvae of the cockroach, *Blattella germanica*. They removed the limb at various times in the insect's molt cycle and showed that regeneration retarded molting. When a limb was amputated early in an instar, the insect regenerated the limb before molting and the instar was lengthened. When a limb was amputated after the prothoracic glands had begun to secrete the molting hormone, the insect molted on schedule without regeneration, and regeneration occurred at the next molt (O'Farrell and Stock, 1953, 1954, 1958; Stock and O'Farrell, 1954; O'Farrell, Stock and Morgan, 1956; and O'Farrell, Stock, Rae and Morgan, 1960). These results were extended by Pohley who examined the delay in molting caused by amputating the antennae of larvae of *Periplaneta americana* (Pohley, 1959) and by extirpating the imaginal wing discs of larvae of the flour moth, *Ephestia kuhniella* (Pohley, 1960). He suggested that the delay in molting was due to the regenerating tissues either inhibiting the growth and differentiation of other tissues or reducing the concentration of molting hormone in the insect.

In addition to these studies of the effects of regeneration on the control of molting, the possible role of the molting hormone in controlling regeneration has also been examined. For example Bodenstein (1955) has analyzed the effects of parabiosis and prothoracic gland transplantation on the regeneration of limbs in adult cockroaches. However, these and other studies focused primarily on the regeneration of cuticular structures which become evident only at a molt and perforce depend on the molting hormone. The present experiments examined the role of the molting hormone in the regeneration of imaginal discs, independently of whether a molt occurred. Our experimental objects were the imaginal wing discs of the larvae of the wax moth, *Galleria mellonella*. We examined regeneration in larvae with actively secreting prothoracic glands, in larvae whose prothoracic glands were removed by ligation, and in larvae without prothoracic glands which received a synthetic molting hormone, ecdysone. The experiments answer the question of whether ecdysone is needed for imaginal disc regeneration and also provide some new information on the mechanism whereby regeneration delays molting.

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MATERIALS AND METHODS

Last instar larvae of *Galleria* from a synchronously molting culture were used for the experiments. The larvae were reared according to the procedure of Sehnal (1966). On the day of ecdysis into the last larval instar, they were collected and kept in petri dishes with food at 30° C and 70% R.H. Such animals pupate 8 or 9 days later, within a day of one another.

Crystalline synthetic α ecdysone was generously provided by Messrs. Hoffman La Roche, Inc. and was dissolved in 10% ethanol to a concentration of 750 μg/ml.

The following surgical operations were performed on 4-day old last instar larvae: (a) Extirpation of one or both imaginal forewing discs or extirpation of all wing discs. (b) Extirpation of right forewing disc and cautery of the wound with a hot needle; this produced a circular burn 2–3 mm in diameter. (c) Sham operated controls; the treatment was like (a) excepting the discs were not extirpated. (d) Ligation of larvae with nylon thread between pro- and mesothorax (to remove the brain and the prothoracic glands). Until six days after the last larval molt this kind of ligation produces "dauer" larvae which survive and respond to tactile stimuli for more than 40 days after ligation. (e) The same ligation as (d) but accompanied by extirpation of the right hind wing disc at specific intervals after ligation. (f) The same operation as (e) but accompanied by injection into each larva of 0.75 μg of synthetic α ecdysone 14 days after ligation. Each group consisted of 20 larvae. A total of 400 larvae were used.

The technique of extirpation of the wings was similar to that developed by Kroeger (*cf.* Schneiderman, 1967). The larvae were etherized for 3 to 4 minutes and placed in a wax-lined dissecting dish filled with insect Ringer solution. On either side of the dorsal midline of the meso- and meta-thoracic segments of the larvae, there is an opaque white spot which marks the position of the tracheae entering the wing disc. A small longitudinal incision was made in this region with a pair of scissors and the imaginal disc was carefully pulled out and then cut off. The cut ends of the skin were lightly pressed together with forceps and the wound sealed with melted paraffin. The operated larvae were then dried on filter paper and transferred to a petri dish with food and kept at 30° C.

The regeneration of the wing discs was examined in the normal larvae at selected intervals, in the ligated groups 30 days after extirpation and in the ecdysone-injected groups 6 days after injection. Simultaneously, the condition of the wing discs on the unoperated side was also recorded.

RESULTS

Normal development of the wing discs

In *Galleria* kept at 30° C, the final larval stadium and prepupal period lasts 8 days for males and 9 days for females. The imaginal wing discs in the 4-day old larva are small flattened epithelial pouches, one on each side of the meso- and meta-thorax, just internal to the epidermis. Each wing analagen contains the epithelial disc tissue which is invaginated into and is covered by a thin layer of epithelium, the peripodial sac, and is supplied with a cluster of tracheae at its base. As the larva grows, the wing anlagen progressively increases in size and develops conspicuous lacunae. In the prepupa (2 to 3 days before pupation) the wing discs undergo a further increase in size and small tracheae migrate into the lacunae. About 12 hours before pupation, the developing wings are everted to the outside of the body to lie beneath the loose larval cuticle that envelops the prepupa. The pupal wings then expand to pupal size. After pupation they tan within a few hours.

Regeneration of extirpated wings in the unligated larva

The process of regeneration of the wing disc was examined at selected intervals in 4-day old larvae in which the right forewing disc was removed. Regeneration of the extirpated wing disc was not evident until 5 days after the operation. However, on the fifth day, a small regenerated wing disc was evident on the extirpated side. It was thin, transparent and about $\frac{1}{8}$ the size of the disc in the unoperated side and had an accumulation of tracheae at its base. It is interesting to note that the unoperated discs and the other tissues of the larva failed to grow or differentiate further and remained in the same stage of development they were when the disc was extirpated. By the seventh day after the operation, the regenerated wing disc had further increased in size and was nearly $\frac{3}{4}$ the size of that on the unoperated side. The unoperated disc also resumed their development. In both the regenerated and control wing disc, lacunae appeared and tracheae began to migrate into them. By 8–9 days after the operation, the regenerated and control wing discs had grown nearly to the same size, differentiated further, and resembled the wing discs of normal larvae immediately before pupation. When the operated larvae pupated, the pupal wings on both sides looked similar and the resulting adults developed normal wings. Commonly, the regenerated wing was about 15% shorter than the wing on the unoperated side.

TABLE I

Results of various surgical operations on 4-day old last instar larvae of Galleria mellonella

Group	Operation	Per cent regenerating	Days to pupate of those regenerating (± = s.d.)	Days to pupate of those not regenerating (± = s.d.)
I	Untreated larvae	—	—	4.5 ± 0.5
II	"Sham" operated controls	—	—	5.6 ± 0.48
III	One forewing disc removed	85	9.6 ± 0.59	6 ± 0.66
IV	Both forewing discs removed	100	13.9 ± 1.2	—
V	All four wing discs removed	100	18.5 ± 1.5	—
VI	Extirpation of one forewing disc plus cautery of the wound	—	—	5.7 ± 0.46

Each group contained 20 larvae.

Effects on pupation of extirpating one, two or four wing discs

The results of extirpating either 1, 2 or 4 wing discs from 4-day old larvae and its effect on their pupation is shown in Table I. The controls for the wing disc extirpation experiments were "sham" operated larvae. All larvae survived the "sham" operation or removal of 1 or 2 of their wing discs. However, when all 4 wing discs were removed 30% of the larvae died as a result of the surgery. The table records the time it took each group of larvae to pupate. Each group consisted of approximately equal numbers of males and females. In almost every group the females pupated on the average a day later than the males; however, since this fact is irrelevant to the present experiments the results for both sexes have been pooled.

Table I shows that the wound caused by the "sham" operation (group II) delayed pupation by one day.

After extirpation of one wing disc (group III), 17 of the 20 larvae regenerated the missing wing disc. Pupation was delayed in these larvae by an average of 5 days and occurred 8–10 days after the operation. The remaining 3 operated larvae failed to regenerate and contained only a small healed stump measuring $\frac{1}{10}$ the size of normal wing disc. These larvae pupated 5, 6 and 7 days after the operation, an average delay of 2 days.

TABLE II

The results of extirpating right hindwing imaginal discs from ligated last instar larvae of G. mellonella

Days after ligation	Per cent of larvae with regenerated wing
0	75
2	75
4	65
6	25
8	15
10	10
14	10

Each group contained 20 larvae.

After removal of both forewing discs (group IV) all of the larvae produced a regenerate and pupated. Pupation of these larvae was delayed by 9 days.

When all wing discs were removed (group V), all of the operated larvae that survived produced a regenerate. Pupation of these larvae was thus delayed by 14 days.

Effect of cautery of the wound on pupation

When the right forewing discs were extirpated from larvae and the overlying epidermal region of the wound was cauterized (group VI, Table I), the larvae molted into pupae 5–6 days after the operation. There was thus only 1 day delay in pupation. The resulting pupae had no right forewings.

Regeneration of wing discs in ligated larva

Four days after the last larval molt larvae were ligated between pro- and meso-thorax to produce "dauer" larvae. Dissection of these larvae at various times after ligation revealed that the further growth of the wing discs and other internal organs had stopped. The results of extirpating wing discs on different days after ligation are recorded in Table II. Each experimental group contained 20 ligated larvae. Larvae were examined for regeneration 30 days after extirpation. In all cases ligation stopped further growth of the unoperated left hand wing disc and other internal organs. However, as the table reveals, when the right hind wing discs were extirpated 0, 2 or 4 days after ligation, more than 65% of the larvae regenerated their right hind wing discs. The remaining 35% failed to regenerate. In the successful cases, dissections showed that the regenerated wing discs had the same shape as the normal regenerated wing discs produced by unligated larvae, but they were thinner than normal regenerates and quite transparent. They were nearly $\frac{3}{4}$ the size of their counterparts in the unoperated side. In most cases, there was accumulation of tracheae in the base of the regenerated disc similar to that seen in normal discs.

When discs were extirpated 6–14 days after ligation, the percentage of larvae in which regeneration occurred decreased markedly from 65% to 10%. The morphology and size of the regenerated wing disc in the 6, 8 and 10 days groups resembled those seen in the 0–4 day groups. However, the few regenerates observed in 14 day group were markedly different. The regenerates were thin and only $\frac{1}{10}$ the size of the wing disc on the unoperated side. There was no further growth in the wing discs on the unoperated side which remained at the same stage of growth as on the day of ligation.

TABLE III

Effects of various treatments on last instar larvae of G. mellonella *ligated for 14 days*

	Per cent regenerating	Per cent molting	Per cent pupating	Per cent remaining larval
Right hind wing disc extirpated on 14th day	10	0	0	100
Right hind wing disc extirpated on 14th day and 0.75 μg of ecdysone injected within 15 min.	65	100	35	0
Right hind wing disc extirpated on 14th day and 0.75 μg of ecdysone injected after 24 hours	0	75	75	25

Each group contained 20 larvae.

Effect of ecdysone on the regeneration of wing discs in ligated larvae

From the experiments reported in section 5 it is clear that ligated larvae gradually lose the ability to regenerate. Is loss of regenerating capacity in these ligated larvae associated with the loss of ecdysone, a decay in the effects of ecdysone, or the absence of some other factor? To answer this, the experiments summarized in Table III were conducted.

Groups of 20 larvae were ligated between pro- and meso-thorax and their right hind wing discs were extirpated 14 days after ligation. One group received no further treatment, (the same group described in the last line of Table II). Only 10% of these larvae regenerated, and these produced a feeble regenerate. A second group of 20 larvae was injected with 0.75 μg of crystalline ecdysone *within 15 minutes after extirpation of the disc* (*i.e.* approximately 10 μg/gm live weight). Within 6 days, 65% of such treated larvae had regenerated their wings. The regenerated wing discs were well-formed and resemble those produced by larvae in which wings were extirpated on the day of ligation. It consisted of disc tissue with a peripodial sac and with tracheae in its base. It was about half the size of the wing disc on the unoperated side. It is also noteworthy that, as a result of ecdysone injection, the wing disc on the unoperated side increased in size and the tracheae began to migrate into the lacunae of the disc tissue. However, development stopped at this point. The ecdysone injection promoted the regeneration of the extirpated disc and also the further development of the unoperated wing disc. The ecdysone also initiated the process of molting. In most cases the old cuticle of the larva could be separated from an underlying new cuticle. In a few cases apolysis occurred, but it was difficult to determine whether or not the underlying epidermis had secreted a new cuticle.

The 35% of ligated larvae which failed to regenerate their wing discs after injection of ecdysone went on to pupate within 4 days of the injection. The wings on the unoperated side grew, differentiated and everted in a normal manner.

A third group of 20 larvae received the injection of 0.75 μg of ecdysone *one day after wing disc extirpation* instead of 15 minutes after extirpation. Different results were obtained. As Table III reveals, the stump healed and no regeneration occurred. Moreover, most of the larvae made some attempt to pupate 3 days after the injection and produced patches of recognizable pupal cuticle. In these larvae the unoperated wing discs grew and differentiated to a stage similar to that seen in normal early prepupa.

DISCUSSION

The necessity of ecdysone for regeneration of imaginal discs of Galleria

The present experiments bear on two questions, namely: the necessity of ecdysone for imaginal disc regeneration and the mechanism whereby regeneration blocks development and molting. A clear answer was obtained to the first question; the second question remains unanswered.

The role of ecdysone in imaginal disc regeneration in *Galleria* was demonstrated in experiments where wing discs were extirpated from larvae whose brains and prothoracic glands had been removed by ligation. When the discs were extirpated from these larvae 0 to 14 days after ligation, the percentage of larvae in which regeneration occurred decreased markedly from 75% to 10%. This defect in ligated larvae was repaired by injecting crystalline α-ecdysone which enabled the larvae to regenerate their extirpated wing discs. These findings lead to the conclusion that regeneration of imaginal discs requires ecdysone. This conclusion explains not only the present results on *Galleria,* but also the results of prothoracic gland transplantation and parabiosis experiments on regenerating adult cockroaches performed by Bodenstein (1955) and are consistent with his prediction that "the loss of regeneration . . . is caused by the absence of thoracic gland hormone" (Bodenstein, 1959, page 3).

The effects of ecdysone on the regeneration of imaginal discs finds some parallel in recent experiments of Oberlander and Fulco (1967) who showed that the increase in size of wing discs of early prepupae of *Galleria* maintained *in vitro* ceased in the absence of α ecdysone and continued in its presence. Further evidence for an effect of ecdysone on imaginal disc regeneration comes from a recent report on the development of imaginal discs in *Drosophila* (Postlethwait and Schneiderman, 1968). When intact imaginal discs of mature *Drosophila* larvae were implanted into adult abdomens, the increase in size of the discs was significantly promoted by injecting an ecdysone. However, neither of these experiments nor the present experiments enable us to decide whether the normal growth of imaginal discs during larval life requires ecdysone.

The ability of ligated larvae to regenerate wing discs up to 10 days after ligation may be due to residual effects of ecdysone that persisted in the larva after ligation. Williams (1968) has suggested such residual or "covert" effects of ecdysone decay over the course of several days. The present experiments reveal that after 14 days the residual effects of ecdysone in ligated *Galleria* larvae either completely disappear or were so slight that the extirpated wing discs failed to regenerate. The persistence of these residual effects of ecdysone may also explain both the observation of Pohley (1961) that mature larvae and early prepupae of *Ephestia* deprived of both brain and prothoracic glands still regenerated wing discs that were removed at the same time the glands were extirpated and also the interpretation that imaginal disc regeneration in *Ephestia* seems to be independent of the molting hormone (*cf.* Wigglesworth, 1965, page 101).

Although ecdysone seemed to be necessary for regeneration in *Galleria*, it was not always effective. In the present experiments 35% of larvae which received 0.75 μg of ecdysone 14 days after ligation failed to regenerate a wing and went on to pupate (Table III). It is significant that this group pupated two days before the 65% that regenerated. To understand this result it is necessary to appreciate the effects of different concentrations of ecdysone on the epidermis and other chitogenous epithelia. Low concentration of ecdysone stimulates growth and cell division whereas high concentration of ecdysone stimulates cuticle secretion and may actually block DNA synthesis, cell division and growth (Krishnakumaran *et al.*, 1967, page 36; Madhavan and Schneiderman, unpublished; Postlethwait and Schneiderman, 1968). In present experiments the 35% of the animals that pupated instead of regenerating a disc after an injection of ecdysone may have been more sensitive to ecdysone and therefore started to secrete a cuticle which, perforce, prevented any regeneration. This interpretation is strengthened by recent observations that when larger doses of ecdysone (10 μg/animal) were injected into ligated *Galleria* larvae with one wing disc extirpated, all of the animals pupated (20 of 20) without regeneration (Madhavan and Schneiderman, unpublished). Apparently ecdysone promotes regeneration only within a certain range of concentrations. Below this range it is ineffective; above this range it promotes prompt cuticle secretion and blocks regeneration.

Also, it is interesting to observe the behavior of the ligated larvae when supplied with 0.75 μg of ecdysone 24 hours *after* extirpation of the wings. In such larvae the dose of ecdysone injected was inadequate to cause pupation: 75% of the larvae made some attempts to pupate, but secreted only occasional patches of pupal cuticle;

the remaining 25% remained larval. Furthermore, no regeneration occurred. Apparently, when the wound healed in the absence of ecdysone, then the healed stump showed a decreased sensitivity to ecdysone when compared with a fresh wound. The enhanced sensitivity of injured tissue to hormones has long been known in the case of juvenile hormones (*cf., e.g.* Schneiderman and Gilbert, 1959) and it may also be true of ecdysone.

Direct and indirect effects of ecdysone on regeneration

From the results discussed above it is evident that the regeneration of imaginal wing discs is promoted by ecdysone. However, the question remains whether this is due to a direct effect of ecdysone on the stump of the disc itself or to an indirect effect of ecdysone on other tissues, or both? Evidence that ecdysone acts directly on the regenerating stump itself comes from recent experiments of Oberlander (1969) who showed that ecdysone promoted DNA synthesis, tracheal migration and disc elongation in wing discs of *Galleria* cultured *in vitro*. This important result argues strongly in favor of a direct action of ecdysone on the discs. It also appears likely that in addition to any effect it has on the disc tissue itself, ecdysone promotes the development of the tracheal system which appears to be necessary for regeneration.

Thus ecdysone may promote regeneration of imaginal disc directly by acting on the imaginal disc cells themselves and indirectly by promoting the growth of the tracheal system.

The role of regenerating tissue in delaying development and molting

One of the most striking features of wing disc regeneration is that it delays both the development of certain other tissues, such as normal wing discs, and the pupal molt. The delay in pupation is not due to the effect of wounding because larvae pupate on time even when they receive several wounds. The delay in pupation is caused by the regenerating wing disc. Such an influence of the regenerating tissue on the molt cycle has been recorded for *Blattella* (O'Farrell, and Stock, 1953), *Periplaneta* (Pohley, 1959) and *Ephestia* (Pohley, 1960, 1965, 1967).

The importance of the regenerating tissue is clearly seen in the experiments reported here in which the regenerating epithelium was destroyed by cautery. Such larvae pupated without delay and without regeneration. Also, in *Galleria,* the delay in pupation increased when several discs were removed and appeared to be roughly proportional to the amount of regenerating tissue: removal of one disc delayed molting by 5 days, removal of two discs delayed it by 9 days, and removal of four discs delayed it by 14 days. Prolonged delay in molting after extirpations of several structures has also been observed in *Blattella* (Stock and O'Farrell, 1954) and in *Ephestia* (Pohley, 1965, 1967). Similar delays in molting and blocks to development can be produced not only by extirpating imaginal discs, but also by implanting an extra disc into the body cavity of a larva (Kroeger, 1958; Muth, 1961). The implant develops a mirror image of itself and, during this process of "doubling," both further growth of host larval structures and pupation are delayed.

This block to both development and molting can be repaired by ecdysone. In the present experiments when a larva with one wing disc extirpated was injected with ecdysone, both the operated disc and the unoperated disc grew and developed simultaneously, whereas in unligated larvae the unoperated disc "waited" until the regenerated disc had "caught up."

Since regeneration, the larval-pupal development of normal wing discs and molting all require ecdysone, one explanation for the blockage of molting is that the regenerate decreases the effective concentration of ecdysone. It might accomplish this (1) by using up the ecdysone present in the larva, (2) by promoting the breakdown of ecdysone, (3) by suppressing but not totally blocking the secretion of the prothoracic glands either directly or indirectly via the brain. An alternative explanation is that the regenerate (4) by nervous or (5) by humoral means changes the responsiveness of other tissues to ecdysone and (6) perhaps promotes the decay of residual or covert effects of ecdysone. For the present, it is not possible to decide between these explanations.

To conclude, the present experiments demonstrate that ecdysone is necessary for the regeneration of imaginal discs in *Galleria;* however, they fail to discern how the regenerating tissue prevents the growth of normal tissues.

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SUMMARY

1. These experiments examined the mechanism whereby regeneration delays development and molting in the waxmoth, *Galleria mellonella*, and also the possible role of the molting hormone in controlling regeneration.

2. Removal of one or more imaginal wing discs from four-day old last instar larvae delayed the further development of unoperated discs and the pupal molt, confirming prior observations on *Ephestia*, *Blattella* and *Periplaneta* by other workers. The delay in molting was proportional to the amount of regenerating tissue: extirpation of one wing disc caused a five-day delay, whereas extirpation of all four discs caused a 14-day delay. When the regenerating epithelium was destroyed by cautery, no delay in molting occurred. Several possible mechanisms for this delay were examined.

3. In another series of experiments four-day old last instar larvae were ligated in such a way as to eliminate both brain and prothoracic glands. When imaginal wing discs were extirpated from these ligated larvae 0 to 14 days after ligation, the percentage of larvae in which the imaginal discs regenerated decreased from 75% to 10%. This defect was repaired by injecting 10 μg/gm of a synthetic molting hormone, alpha ecdysone, immediately after extirpating the wing disc. This treatment enabled the larvae to regenerate the missing disc.

4. It is concluded that regeneration of imaginal discs in *Galleria* requires ecdysone.

5. Additional experiments indicated that ecdysone promoted regeneration only within a certain range of concentrations: a concentration of 10 μg/gm promoted regeneration whereas 100 μg/gm promoted the prompt secretion of cuticle and blocked regeneration.

6. Ecdysone was less effective in promoting regeneration if the wound healed before it was applied. Thus injecting 10 μg/gm *immediately* after wing disc extirpation promoted regeneration, whereas an identical injection *24 hours after* extirpation failed to promote regeneration. This suggests that injured tissues may be more sensitive to ecdysone.

7. Arguments were advanced that ecdysone promoted regeneration of *Galleria* wing discs directly by acting on the imaginal discs themselves and perhaps indirectly by promoting the growth of the tracheal system.

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RYNKATORPA PAWSONI N. SP. (ECHINODERMATA: HOLOTHUROIDEA) A COMMENSAL SEA CUCUMBER[1]

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An angler fish, *Gigantactis macronema* Regan, was collected September 12, 1968 by the VALERO, scientific vessel of the University of Southern California, in a net haul between 500 and 1050 fathoms, 19.5 miles southeast of Head Light on San Clemente Island off the coast of Southern California. Four small, gray, nearly cylindrical organisms were attached to one side of the fish grouped near one another. At first it was thought that these might be helminths, but study of three mounted whole and one serially sectioned revealed that they were sea cucumbers. The whole mounts were stained with Mayer's paracarmine and the serial sections with Mallory's triple. This apparently is a new ecological association for echinoderms. A note has been published on this association (Martin, 1969). This species is named in honor of Dr. Pawson, Smithsonian Institution, in recognition of his many contributions to the knowledge of holothurians.

DESCRIPTION

Order Apodida Brandt, 1835
Family Synaptidae Burmeister, 1837
Rynkatorpa Rowe and Pawson, 1967
Rynkatorpa pawsoni n. sp.

Body subcylindrical, gray (when fixed in alcohol), 5.18 to 7.14 mm long and 1.75 to 2.52 mm in maximum width. Body surface irregular with small lobes and indentations. Raised clusters of nuclei in circles or ridges scattered over body surface. Tube feet, anchors, plates, and respiratory tree lacking. Twelve digitiform, muscular, retractable tentacles, each with 2 digits. Pharynx with twelve ridges projecting into its lumen. Digestive tract tubular, looped, with anus at posterior end of body. Intestinal lining smooth in some areas and multi-ridged in others. Five longitudinal muscle bands containing thick and thin fiber bundles are interconnected by circular muscle fibers. Ring canal near bases of tentacles with one polian vesicle and one stone canal, approximately opposite one another. Nerve ring near bases of tentacles with a nerve to each tentacle and to each longitudinal muscle band. Small lobed mass of cells seen in one specimen in anterior third of body of unknown function. One specimen has a branched structure in the posterior part of the body that might be a gonad.

Attached by posterior end to the fish host: *Gigantactis macronema* Regan. Locality: Near coast of Southern California Holotype E11154; paratype E11155 U. S. Nat'l. Mus.

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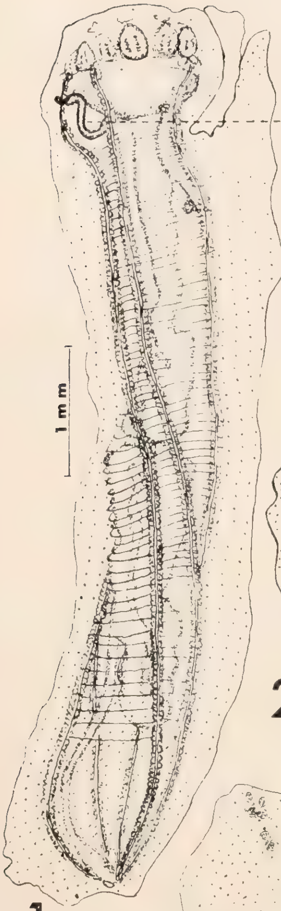
[1] Supported by N. S. F. GB 6962.



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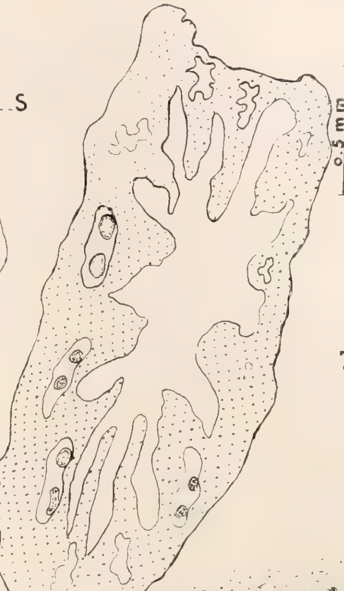

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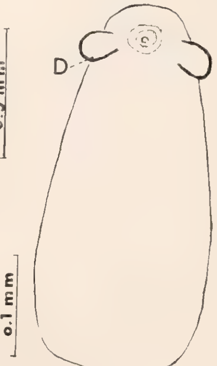

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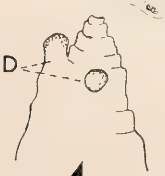

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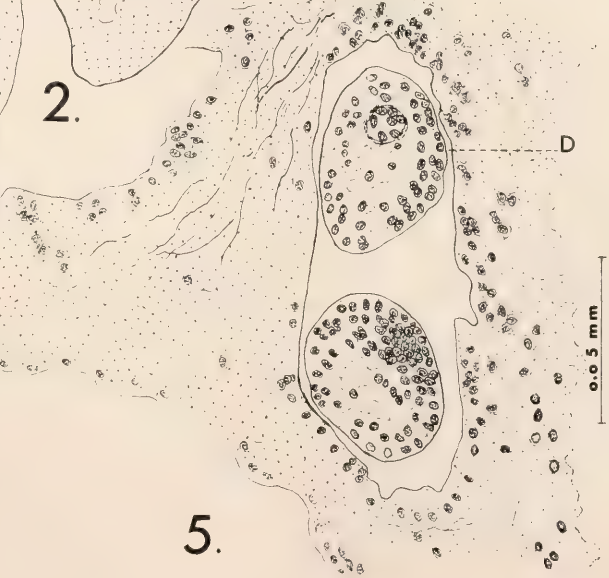

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FIGURE 1. Lateral view of entire specimen, stone canal (S). All figures drawn with the aid of a camera lucida unless otherwise indicated.

FIGURE 2. Section through body near anterior end. Cross sections of some digits are evident in tentacular sacs. Twelve ridges project into digestive tract.

FIGURE 3. Diagram of an extended tentacle with digits (D).

FIGURE 4. Lateral view of partly extended distal part of tentacle bearing two digits (D).

FIGURE 5. Area surrounding a tentacle sac containing two digits (D).

Further descriptive details are as follows. The body surface is very irregular with identations, folds and protrusions of various shapes. Some of the thinner folds are composed of only two layers of epidermis while most contain an additional fibrous layer. The thin cytoplasmic epidermis contains numerous nuclei, usually in single file. Cell walls were not observed even under the oil immersion lens and therefore the epidermis is believed to be syncytial. However, electronmicrographs might reveal cell walls. Except for the clumping of nuclei in scattered knobs, ridges and crescents, there is no apparent specialization of the epidermis in different regions of the body. The thickness of the epidermis usually varies from 3 μ to 9 μ except at the knobs or other raised areas where it may reach 30 μ.

The fibrous layer or dermis, underlying the epidermis, forms the bulk of the body wall. The fibers, presumably collagenic, form a dense network ramifying in all directions. Here and there are knot-like masses where fibers converge. Some fibers are only 0.3 μ but others are 3.0 μ in thickness. The fibrous layer varies in thickness from 20 μ to 32 μ. Scattered in the fibrous layer are cells, probably fibrocytes.

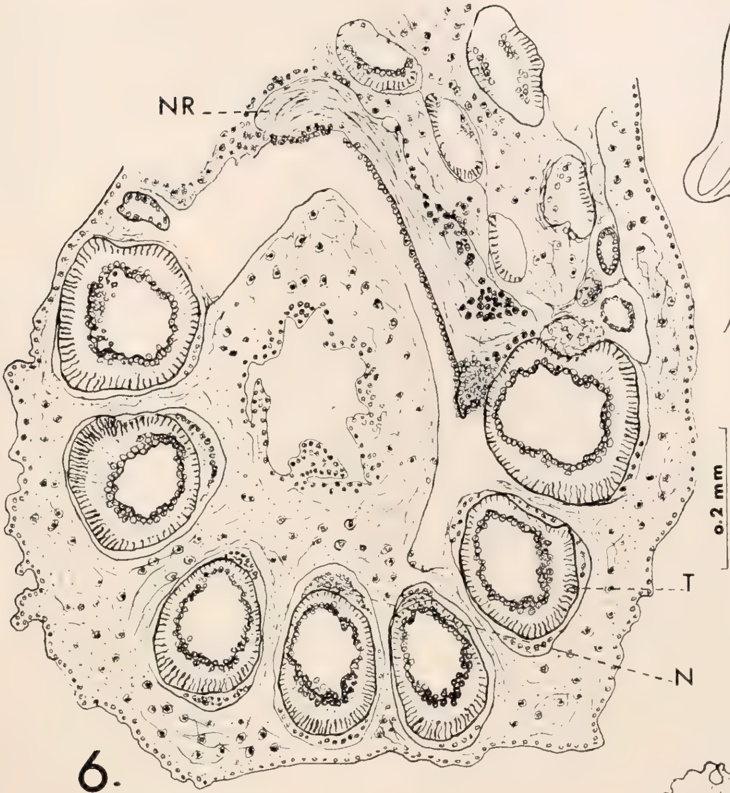
Some of the five longitudinal muscle bands decrease in size at certain levels of the body but are well developed anterior and posterior to these levels.

The coelomic lining is a delicate layer only 1.6 μ thick where it thins out between nuclei but it is 6 μ at nuclear locations. This layer forms thin mesenteries from 3 μ to 6 μ thick. In the anterior and posterior parts of the body, the mesenteries include a few muscle fibers. Mesenteries are more numerous in the anterior body region. The mesenteries attach to ridged modifications of the wall of the digestive tract. Most such ridges are nearly circular in cross section with attenuated bases where they join the digestive tract. They include an extension of connective tissue fibers from the wall of the digestive tract.

The branched structure seen in the posterior part of the body of one specimen and suggested as a possible gonad is in an unusual position because holothurians generally have gonads in the anterior part of the body.

The stone canal wall is packed with nuclei and has a sparse supply of connective tissue fibers. It is about 16 μ thick. The outside dimensions of a cross section of the stone canal are approximately 44 μ by 68 μ. The lumen of the canal is about 6 μ in diameter.

The mouth is irregular in outline with twelve longitudinal ridges, corresponding in position to the tentacles, that project into the lumen. It leads into the pharynx which has a relatively thick wall that contains some muscle and connective tissue fibers. The most elaborate infoldings of the digestive tract lining occur in the pharynx. These may have central columns reinforced by fibers and support as many as six folds on each side. The esophagus is thin-walled and also has folds extending into its lumen except where it is expanded and the lining is smooth. The stomach has the thickest wall and it appears to be mainly glandular with very poor representation of muscle and connective tissue fibers. It is richly supplied with nuclei some of which are twice as large as others. The lining of the stomach is smooth where this organ is expanded but is longitudinally ridged in other areas. The intestine is nearly circular in cross section and it contains numerous projections into the lumen. The posterior part of the intestine has



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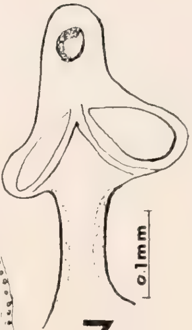

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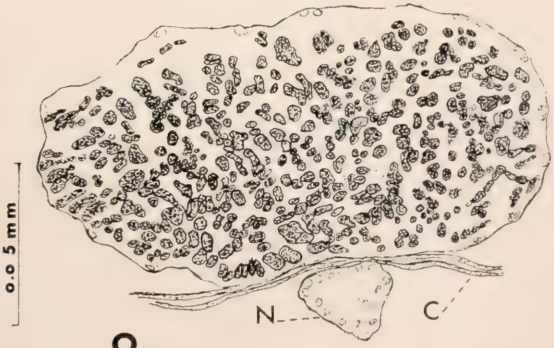

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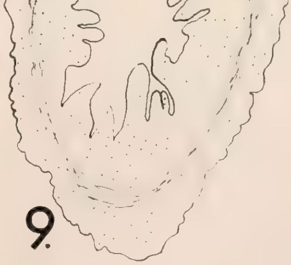

9.

FIGURE 6. Section through tentacle region showing cross sections of tentacles (T) with muscle fibers; nerve (N) supplying tentacle and nerve ring (NR).

FIGURE 7. Lateral view of polian vesicle.

FIGURE 8. Cross section of longitudinal muscle band, associated circular muscle fibers (C) and nerve (N).

FIGURE 9. Cross section of digestive tract showing ridged lining.

more muscle fibers than any other part of the digestive tract. Here, approximately one-half the thickness of the wall is composed of muscle fibers.

The nerve ring and longitudinal nerve cords have the neuronal nuclei located mainly on or near the surface where they form almost a continuous layer.

The walls of the tentacles are richly supplied with muscle fibers especially on the side nearer to the body surface. There is a thin fiber layer immediately peripheral to the muscle layer. The tentacles and digits contain lumina.

Discussion

The lack of anchors and plates in this species may be natural or may be due to treating the specimens with weak HCl in the destaining process. Their absence makes it difficult to decide if the species should be placed in the genus *Protankyra* Östergren, 1898, *Rynkatorpa* Rowe and Pawson, 1967, or in a new genus. Rowe and Pawson (1967) transferred several species from *Protankyra* to *Rynkatorpa* mainly on differences in anchors and plates. Two of those transferred, *R. duodactyla* (H. L. Clark, 1907) and *R. bicornis* (Sluiter, 1901), are like *R. pawsoni* in having two digits on each of the twelve tentacles. However, *R. bicornis* has four or five stone canals and *R. duodactyla* has two polian vesicles. Both *R. bicornis* and *R. duodactyla* are bottom-dwelling species. Because of the similarities of twelve tentacles, each with two digits, the species *pawsoni* is provisionally placed in the genus *Rynkatorpa*.

In the cross section of a longitudinal muscle band (Fig. 8) there appear to be fibers of different diameters. However, the larger units are either clusters or oblique or transverse fibers as has been reported for *Stichopus mollis* by Freeman and Simon (1964). Baccetti and Rosati (1968) examined the ultrastructure of muscles in *Holothuria tubulosa* and suggested that they contain paramyosin, the first reported occurrence in the phylum Echinodermata of the "catch" type of muscle. Paramyosin has been found in lamellibranchs, gastropods, annelids, and possibly arthropods.

Although *R. pawsoni* is firmly attached to the bathypelagic host's rough skin and small dermal denticles, there seems to be little or no invasion of host tissues. Hence, the association is believed to be commensal with the cucumber benefitting by being taxied about with no effect upon the host. How the cucumber contacts the fish host remains unknown. Some cucumbers can swim (Hansen and Madsen, 1956) and perhaps *R. pawsoni* can.

The *Gigantactis macronema* from which the cucumbers were removed is one of the largest (about 325 mm, standard length) ever captured. Bertelsen (1951) mentions that the type specimen of *G. macronema* is 133 mm long. The illicium (slender lure) of the present specimen is over three and one-half times the body length. Bertelsen also stated that these fish are quite widely distributed and that they generally are captured at depths between 500 and 2000 meters.

I wish to acknowledge the help of my assistant, Mr. Kevin Springer, for making the serial sections; Dr. Russel Zimmer for suggesting that the specimens might be holothurians; and Dr. David L. Pawson for critically reading the manuscript.

SUMMARY

A new species of sea cucumber is described from the Pacific Ocean off Southern California. It is provisionally assigned to the genus *Rynkatorpa* as *R. pawsoni*. Four specimens were found attached to the side of a bathypelagic fish, *Gigantactis macronema* Regan. There was no apparent invasion of host tissue so the association is believed to be commensalism. This is the first description of a commensal echinoderm.

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LARVAL DEVELOPMENT OF *BATHYNECTES SUPERBA* (COSTA) REARED IN THE LABORATORY[1]

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Larvae of several portunid species have now been described to varying extents by laboratory culture studies, including *Portunus puber* (egg to juvenile), *P. depurator* (egg to Zoea III), *Carcinus maenas* (egg to Zoea IV) by Lebour (1928), and *Callinectes sapidus* (egg to Zoea IV, Hopkins, 1943, 1944; egg to juvenile, Costlow and Bookhout, 1959). The first zoea of several additional species have been obtained from the egg, including *Bathynectes longipes,* several *Portunus* species, and *Polybius henslowi* (Lebour, 1928, 1931, 1944, 1950). Later stages of several species were described from larvae collected from the plankton, held through one or more molts and arranged in series up to a recognizable juvenile or adult form (Lebour, 1928,1944; Aikawa, 1929, 1933, 1937). Based on this work it is possible to identify many larvae in this family at least to genus. Early work does not provide information necessary to separate *Bathynectes* from other portunids because only the first stage was known and that incompletely described by present day criteria; thus Lebour (1928, 1931) was unable to distinguish *Bathynectes, Polybius,* and *Portunus.*

The present study was undertaken to determine the number of larval stages in *Bathynectes,* and to describe each larval stage in detail based on laboratory reared specimens. Criteria for distinguishing *Bathynectes* from other Portunidae were sought.

MATERIALS AND METHODS

An ovigerous *Bathynectes superba* was collected off the Virginia coast during a routine cruise in February, 1968, and brought to the laboratory in a small amount of water. The temperature at the collection station was about 10 C; the crab was maintained at this temperature during transit. The salinity was approximately 34‰. Water used for transporting the crab contained a considerable amount of mucous and fish scales, but the crab was alive and soon became active after being placed in a battery jar with clean ocean water at about 10 C.

The eggs were nearly ready to hatch, with eye spots, a beating heart, and larval pigmentation visible within each egg capsule. Some eggs were removed from the female and cultured using the procedures developed by Costlow and Bookhout (1960). Temperature was maintained at 10 C during incubation of the eggs. The female with the remainder of the egg mass was maintained in about 5 liters of water with daily water changes.

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After two days, weak pre-zoeae hatched from the egg mass on the female. During the following two days, Zoea I were obtained both from the female and from the incubated eggs. A total of 221 zoeae were segregated in compartmented plastic boxes, 2 zoeae per compartment. In addition, a large number (500-1000) were placed in mass culture in about 1 liter of water. Cultures were maintained in a cold room at 13 C or less for most of the culture period. Maximum temperature for the period was slightly above 16 C for one day. Nauplii of *Artemia salina* were provided as food. During the early part of the culture period, water collected at the same time and location as the ovigerous crab was used, with transfers every second day. On the fifteenth day, it became necessary to use water from Wachapreague Inlet, Virginia, concentrated to 34‰ by freezing and decanting. There was no marked change in mortality rate at this time.

Approximately 30 zoeae from the mass culture were preserved in 70% ethanol with 10% glycerol for detailed anatomical study along with dead specimens from the individual culture. Specimens of each stage were measured, and certain obvious details of setation and ornamentation noted. Figures of whole specimens and dissected appendages were made, and from these, plus notes made on several dissected specimens, descriptions of each stage were written and subsequently checked against additional specimens. All dissections were performed under 85% lactic acid. Measurements taken were: distance from tip of rostrum to tip of dorsal spine, length of rostrum, dorsal spine, and lateral spines, carapace length (tip of rostrum to posterior margin of carapace along mid dorsal line), abdominal length, and total length (carapace length plus abdominal length). The latter measurement is not exactly the "true" total length, but is the best possible approximation since the zoeae, living and dead, are so curved that no direct measurement is possible.

The following abbreviations are used throughout the paper: A 1 = antennule, A 2 = antenna, Mn = mandible, Mx 1 = maxillule, Mx 2 = maxilla, Mxp 1 = first maxilliped, Mxp 2 = second maxilliped, Mxp 3 = third maxilliped, P 1 to 5 = pereiopods 1 to 5, Pl 2 to 5 = pleopods 2 to 5, U = uropod.

RESULTS

Five zoeal stages were obtained, the last one being terminal as judged by degree of development of pereiopod and pleopod buds. Unfortunately the megalopa was not obtained. Mean intermolt duration for each stage which molted was calculated from individual culture data: Zoea I, 8 days, Zoea II, 6 days, Zoea III, 7 days, Zoea IV, 11 days. One larva died 17 days after reaching Zoea V, without molting to the megalopa; most Zoea V's died after only 5 or 6 days.

Zoea I (Fig. 1)

Carapace (Fig. 1a) with rostral, dorsal, and lateral spines. Rostrum straight, strongly depressed. Dorsal spine long, strongly curved. Lateral spines short, strongly depressed, with very broad base giving carapace a flared appearance; lateral carapace margin with definite lobe below and behind eye. Eyes swollen, immobile. Abdomen (Fig. 1b) with 5 somites and telson. Two minute hairs

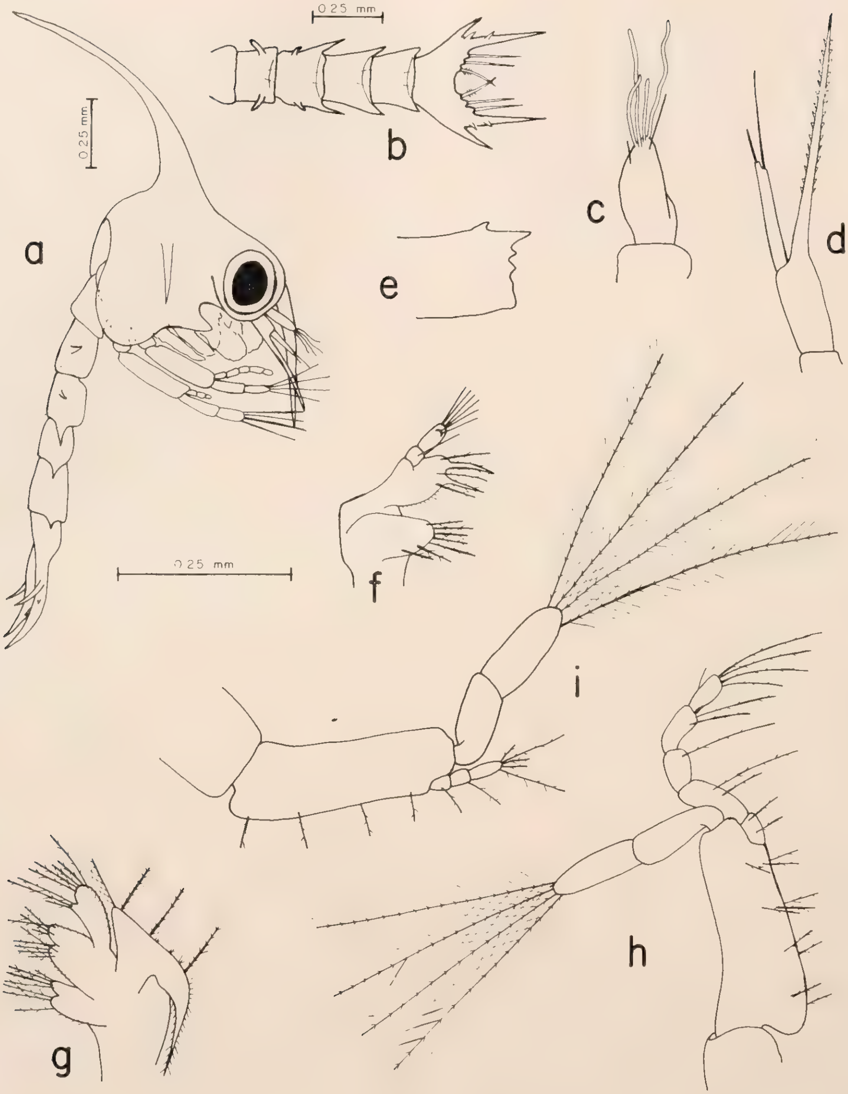


FIGURE 1. Zoea I of *Bathynectes superba,* a. lateral view, b. dorsal view of abdomen, c. antennule, d. antenna, e. mandible, f. maxillule, g. maxilla, h. first maxilliped, i. second maxilliped.

dorsally on second through fifth abdominal somites. Somites 3, 4, and 5 with long posterolateral spines, shortest on somite 5. Somites 2 and 3 with lateral knobs. Telson bifurcate, each furca with a long lateral spine curving dorsally plus 2 minute spines, 1 dorsal and 1 lateral. Furcal tips curved dorsally. Three median setae, articulated, with irregular setulation. No anal spine.

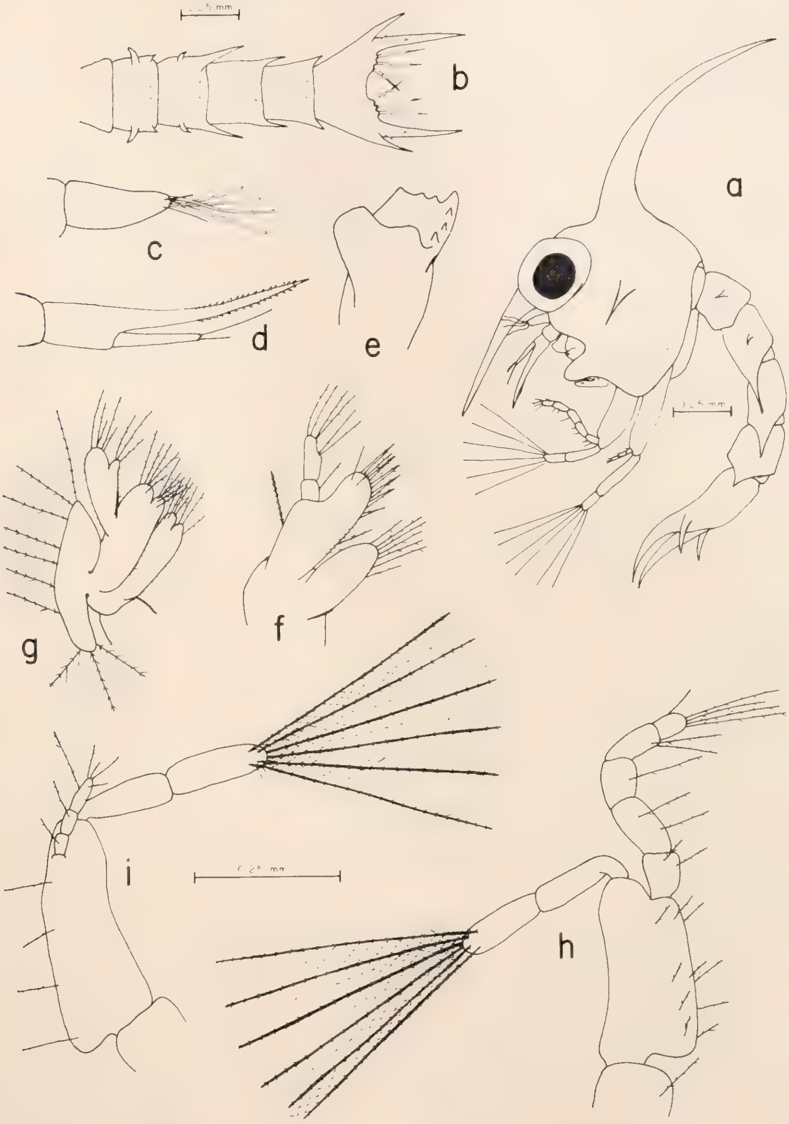


FIGURE 2. Zoea II of *Bathynectes superba*, a. lateral view, b. dorsal view of abdomen, c. antennule, d. antenna, e. mandible, f. maxillule, g. maxilla, h. first maxilliped, i. second maxilliped.

A 1 (Fig. 1c) Uniramous, with 4 terminal aesthetascs plus 2 setae.

A 2 (Fig. 1d)—Ventral peduncular spine with cuneate setules in 2 rows. Spine twice as long as exopod; exopod with 2 terminal setae, 1 long, 1 short. No endopod.

Mn (Fig. 1e)—Incisor process only, with 4 terminal teeth plus 1 tooth on posterior margin.

Mx 1 (Fig. 1f)—Coxal endite with 7 setae; basal endite with 5 setae; fringed with fine hairs on proximal margin. Endopod 2-segmented, with 4 terminal and 2 subterminal setae; 1 seta on proximal segment.

Mx 2 (Fig. 1g)—Coxal endite bilobed with 3 setae on proximal lobe, 4 on distal lobe; basal endite bilobed with 5 setae on proximal lobe, 4 on distal lobe; both endites fringed with fine hairs. Endopod unsegmented with 5 terminal setae and 3 setae on subterminal knob; margins fringed with fine hairs. Scaphognathite with proximal and distal lobes, 4 plumose setae; proximal lobe drawn out into long plumose projection.

Mxp 1 (Fig. 1h)—Coxa nude, basis with 10 setae on inner margin. Endopod with 2, 2, 1, 2, 5 setae on segments 1 to 5. Exopod 2-segmented with 4 terminal plumose setae.

Mxp 2 (Fig. 1i)—Coxa nude, basis with 4 setae on inner margin. Endopod with 1, 1, 5 setae on segments 1 to 3. Exopod 2-segmented with 4 terminal plumose setae.

Mxp 3—Very minute biarmous rudiment, smaller than Mxp 2 coxa.

P 1 to 5—Minute ridges the only visible rudiments.

Zoea II (Fig. 2)

Carapace, abdomen, and telson unchanged (Fig. 2ab). Eyes stalked.

A 1 (Fig. 2c)—Unchanged from Zoea I.

A 2 (Fig. 2d)—Unchanged from Zoea I.

Mn (Fig. 2e)—Incisor process with 4 terminal teeth and 3 teeth on posterior margin. Molar process now present.

Mx 1 (Fig. 3f)—Coxal endite with 7 setae; basal endite with 9 setae, fringed with fine hairs. Endopod with 4 terminal and 2 subterminal setae; 1 seta on proximal segment. A long plumose seta now present on basis proximal to endopod (epipodital hair).

Mx 2 (Fig. 2g)—Coxal endite bilobed with 3 setae on proximal lobe, 4 on distal; basal endite bilobed with 5 setae on each lobe; both endites fringed with fine hairs. Endopod with 5 terminal setae plus 3 setae on subterminal knob; both margins fringed with fine hairs. Scaphognathite with 10 plumose setate; proximal lobe not produced into a long projection.

Mxp 1 (Fig. 2h)—Coxa with 1 seta, basis with 10 setae on inner margin. Endopod with 2, 2, 1, 2, 5 setae on segments 1 to 5. Exopod with 6 terminal setae.

Mxp 2 (Fig. 2i)—Coxa nude, basis with 4 setae on inner margin. Endopod with 1, 1, 5 setae on segments 1 to 3. Exopod with 6 terminal plumose setae.

Mxp 3—Slightly larger biramous rudiment.

P 1 to 5—Buds enlarged, still smaller than Mxp 3 rudiment.

Zoea III (Fig. 3)

Carapace (Fig. 3a) unchanged except for thickening of dorsal spine. Abdomen (Fig. 3b) with 6 somites plus telson. Ornamentation unchanged from Zoea I except for slightly increased length of lateral spines on somite 4 and addition of

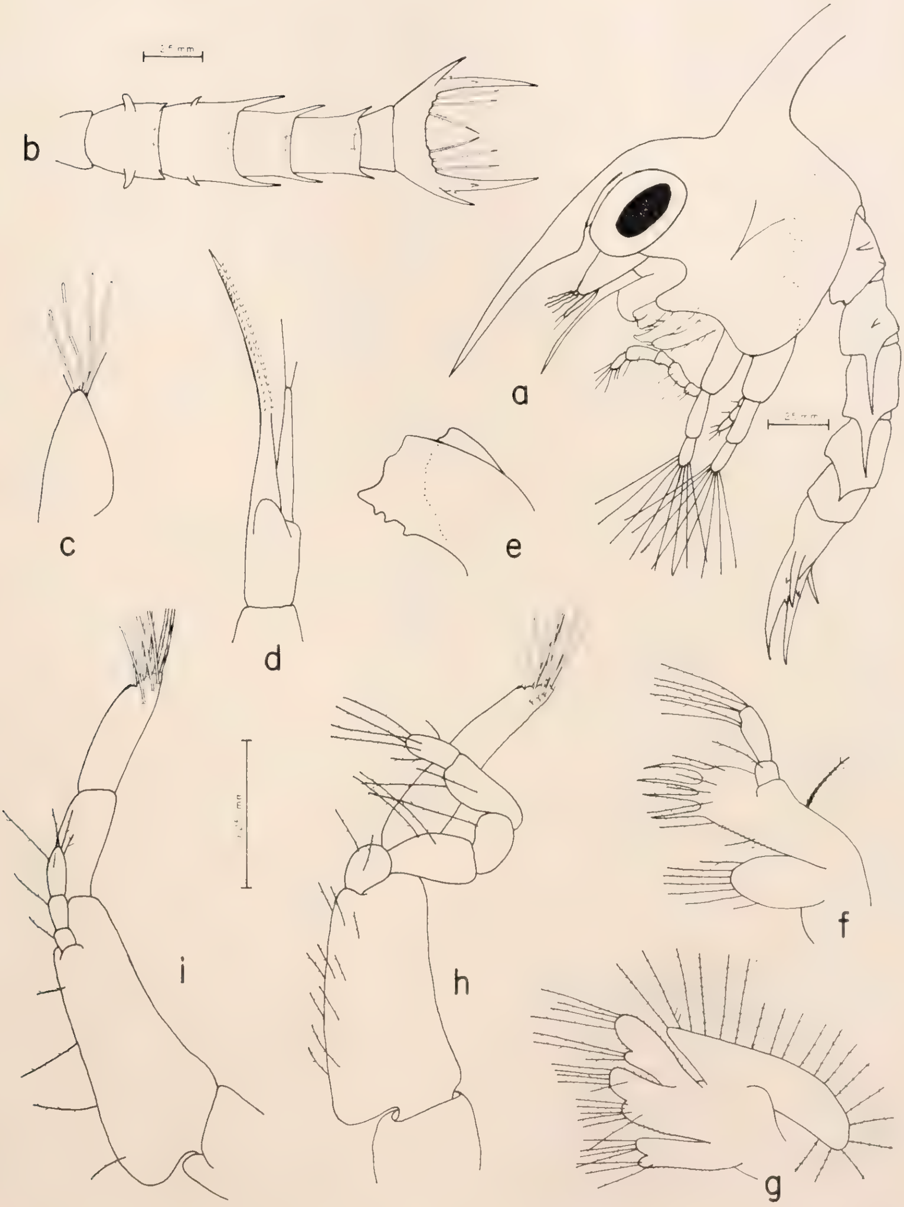
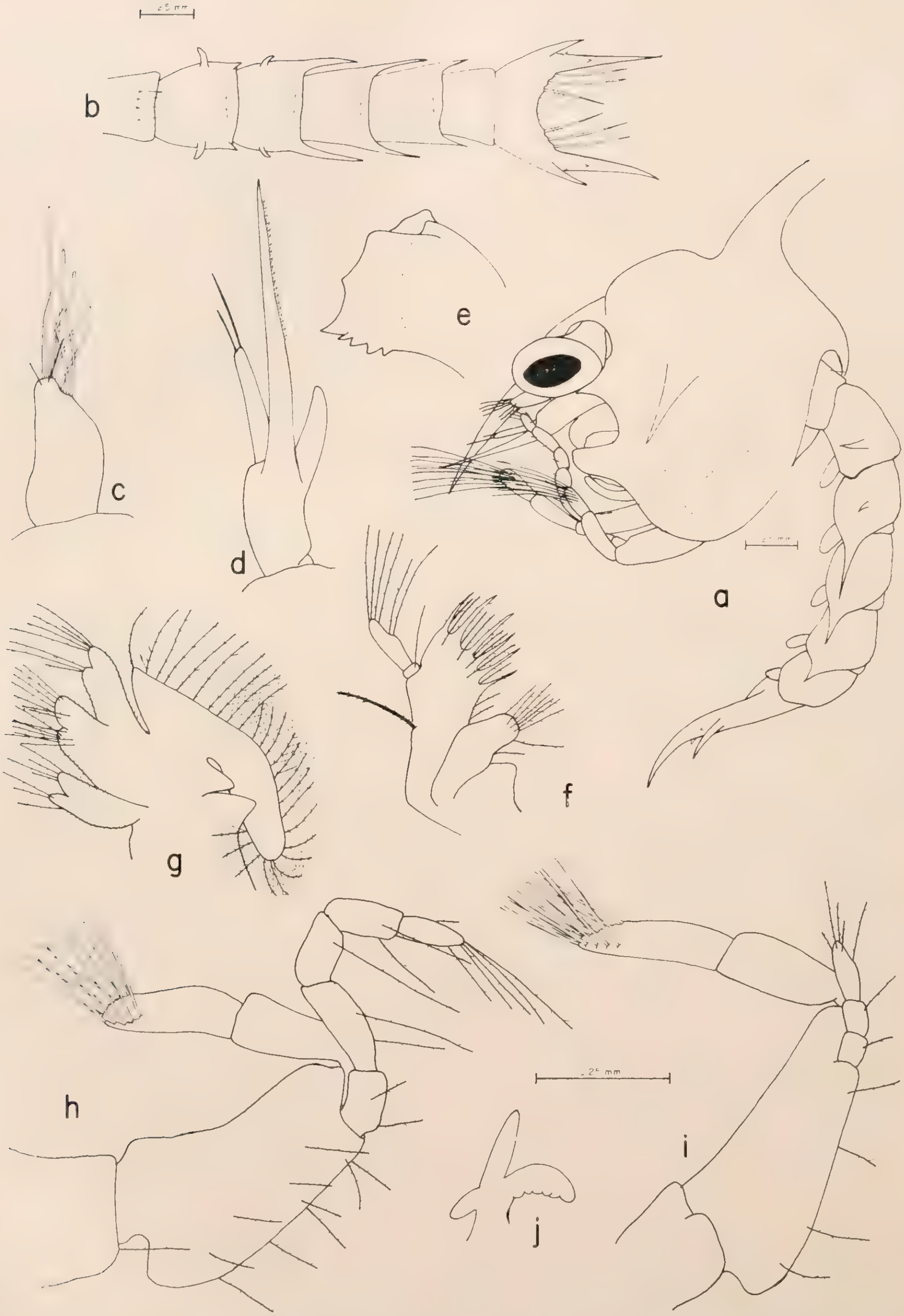


FIGURE 3. Zoea III of *Bathynectes superba*, a. lateral view, b. dorsal view of abdomen, c. antennule, d. antenna, e. mandible, f. maxillule, g. maxilla, h. first maxilliped, i. second maxilliped.


.5 mm
b
c
d
e
a
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f
g
h
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j
i

FIGURE 4. Zoea IV of *Bathynectes superba,* a. lateral view, b. dorsal view of abdomen, c. antennule, d. antenna, e. mandible, f. maxillule, g. maxilla, h. first maxilliped, i. second maxilliped, j. third maxilliped.

4 dorsal hairs on somite 1. Pleopod anlagen sometimes visible beneath exoskeleton. Telson still broad, with a pair of very short setae between the original three pairs.

A 1 (Fig. 3c)—Unchanged from Zoea I.

A 2 (Fig. 3d)—Unchanged from Zoea I except for addition of small endopod bud.

Mn (Fig. 3e)—Incisor process with 3 terminal teeth plus 3 teeth along posterior margin. Molar process with several teeth.

Mx 1 (Fig. 3f)—Coxal endite with 7 setae; basal endite with 10 setae, fringed with fine hairs. Endopod with 4 terminal and 2 subterminal setae; 1 seta on proximal segment. Epipodital hair present.

Mx 2 (Fig. 3g)—Coxal endite bilobed with 3 setae on proximal lobe, 4 on distal; basal endite bilobed with 6 setae on proximal lobe, 5 on distal; both endites fringed with fine hairs. Endopod with 5 terminal setae plus 3 setae on subterminal knob; margins fringed with fine hairs. Scaphognathite with 19 plumose setae, 6 long setae on distal lobe, 13 short setae on proximal lobe.

Mxp 1 (Fig. 3h)—Coxa with 1 seta, basis with 10 setae on inner margin. Endopod with 2, 2, 1, 2, 6 setae on segments 1 to 5. Exopod with 8 terminal plumose setae.

Mxp 2 (Fig. 3i)—Coxa nude, basis with 4 setae on inner margin. Endopod with 1, 1, 5 setae on segments 1 to 3. Exopod with 8 terminal plumose setae.

Mxp 3—Still a small biramous rudiment.

P 1 to 5—Rudiments slightly larger than Mxp 3.

Zoea IV (Fig. 4)

Carapace (Fig. 4a) with shield area prominently thickened; lateral marginal lobes prominent; 6 fine setae on posterolateral area near margin, on inner surface, directed medially (not readily visible, unfigured). Abdominal somites unchanged (Fig. 4b). Telson with a second pair of short setae medially.

A 1 (Fig. 4c)—Uniramous, with 4 aesthetascs and 2 setae terminally, 3 aesthetascs subterminally.

A 2 (Fig. 4d)—Unchanged from Zoea III except endopod bud considerably larger, about ½ exopod.

Mn (Fig. 4e)—Unchanged from Zoea III.

Mx 1 (Fig. 4f)—Coxal endite with 9 setae; basal endite with 16 setae, fringed with fine hairs. Endopod with 4 terminal and 2 subtermial setae; 1 seta on proximal segment. Epipodital hair present.

Mx 2 (Fig. 4g)—Coxal endite bilobed with 3 setae on proximal lobe, 4 on distal; basal endite bilobed with 7 setae on each lobe; both endites fringed with fine hairs. Endopod with 5 terminal setae and 3 setae on subterminal knob; margins fringed with fine hairs. Scaphognathite with 27 plumose setae, 10 long setae on distal lobe, 17 short setae on proximal lobe.

Mxp 1 (Fig. 4h)—Coxa with 1 seta, basis with 10 setae on inner margin. Endopod with 2, 2, 1, 2, 6 setae on segments 1 to 5. Exopod with 10 terminal plumose setae.

Mxp 2 (Fig. 4i)—Coxa nude, basis with 4 setae on inner margin. Endopod with 1, 1, 5 setae on segments 1 to 3. Exopod with 10 terminal plumose setae.

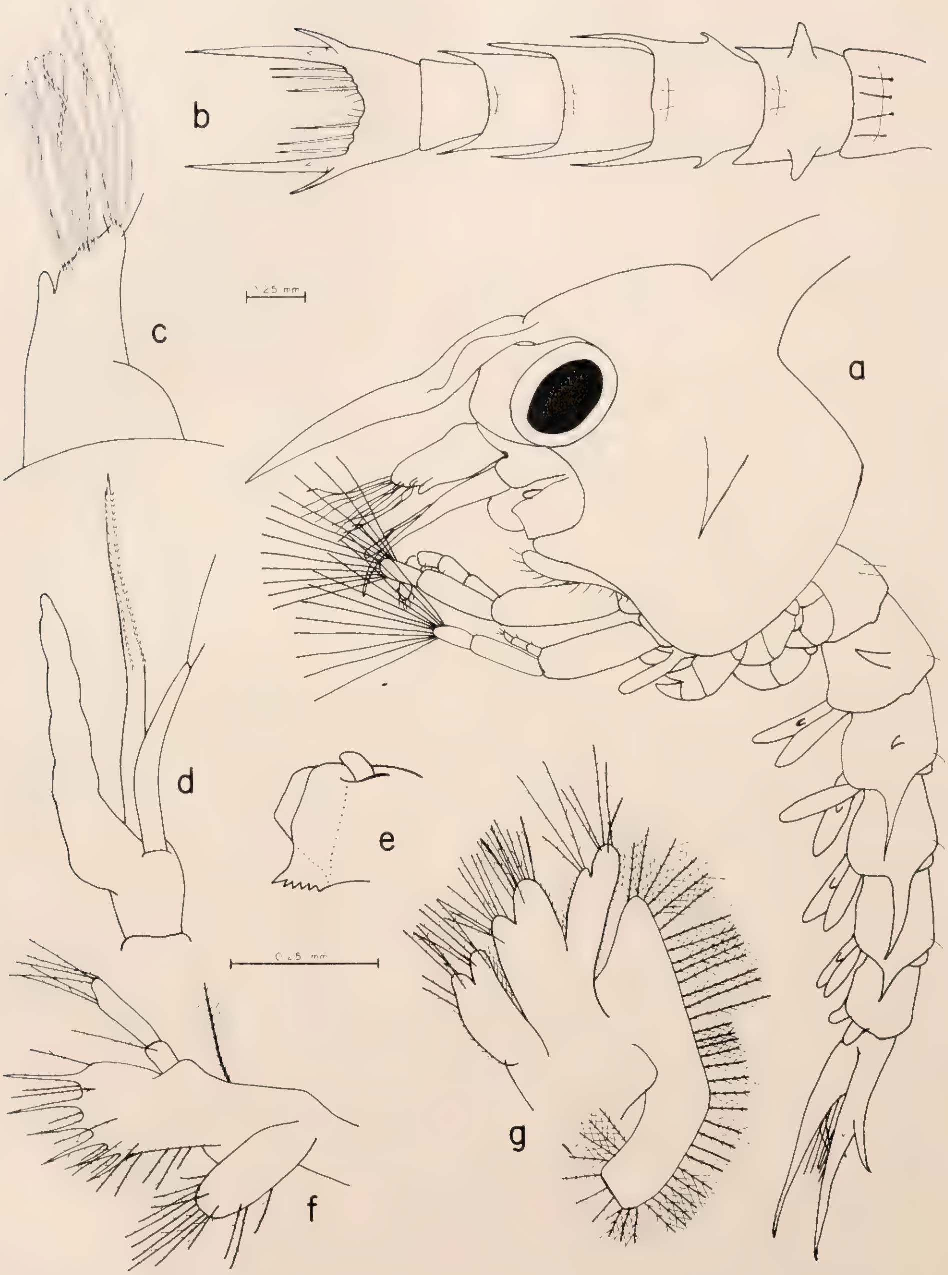
b
c
0.25 mm
a
d
e
0.25 mm
g
f

Figure 5. Zoea V of *Bathynectes superba,* a. lateral view, b. dorsal view of abdomen, c. antennule, d. antenna, e. mandible, f. maxillule, g. maxilla.

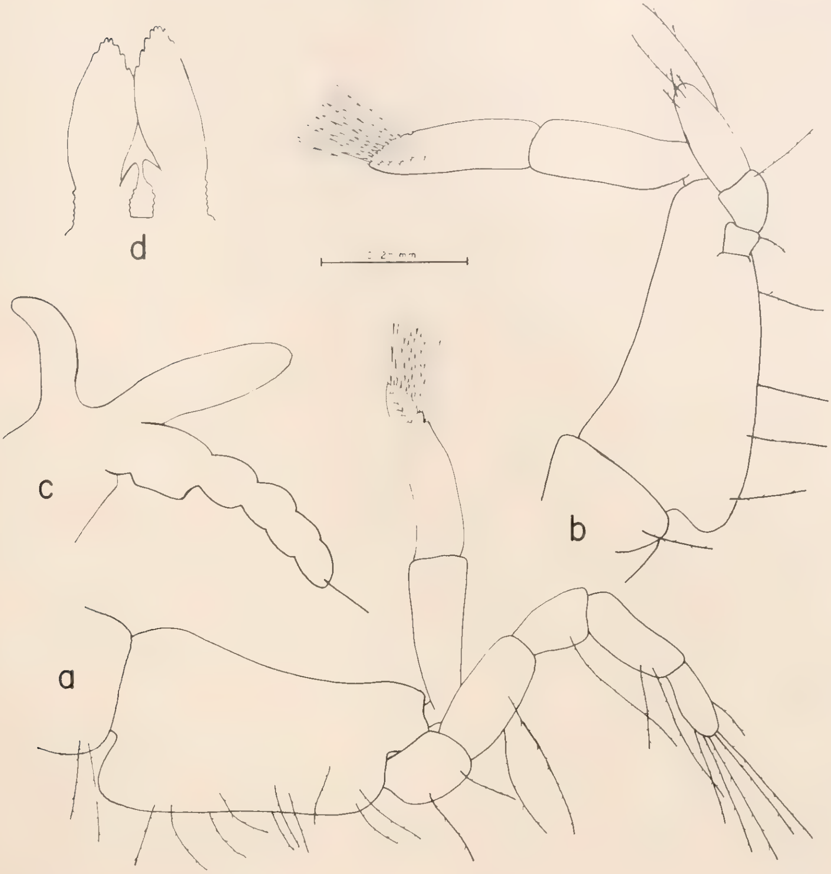

FIGURE 6. Zoea V of *Bathynectes superba,* a. first maxilliped, b. second maxilliped, c. third maxilliped, d. pleopod buds, abdominal somite 4.

Mxp 3 (Fig. 4j)—Rudiment triramous; endopod wrinkled, curved medially; exopod straight; epipod curved posteriorly into branchial chamber. No setae on rami.

P 1 to 5—Greatly enlarged; pseudosegmented; P 1 with biramous tip or chela. P 5 hidden behind P 2 to 4.

Pl 2 to 5 (Fig. 4a)—Small uniramous buds; medially located on somites.

U—Small uniramous bud; laterally positioned on somite 6.

Zoea V (Figs. 5 and 6)

Carapace and abdomen (Fig. 5a, b) unchanged from Zoea IV; 8 setae on posterolateral region of carapace.

A 1 (Fig. 5c)—Biramous, 4 aesthetascs and 2 setae terminally, 8 aesthetascs subterminally, 5 distally, 3 proximally. Inner ramous present as small subterminal palp.

A 2 (Fig. 5d)—Unchanged from Zoea IV except endopod bud about $\frac{3}{4}$ spine.

Mn (Fig. 5e)—Incisor process with no terminal teeth but 8 teeth along posterior margin. Molar process with several teeth. Palp present, unsegmented.

Mx 1 (Fig. 5f)—Coxal endite with 13 setae; basal endite with 19 setae. Endopod with 4 terminal and 2 subterminal setae; 1 setae on proximal segment. Epidopdital hair present.

Mx 2 (Fig. 5g)—Coxal endite bilobed with 5 setae on each lobe; basal endite bilobed with 8 setae on proximal lobe, 9 on distal; both endites fringed with fine hairs. Endopod with 5 terminal and 3 subterminal setae; margins fringed with fine hairs. Scaphognathite with 42 plumose setae, 14 long setae on distal lobe, 28 short setae on proximal lobe.

TABLE I

Zoeal dimensions with mean, observed range, and number measured for each stage

Zoea	Rostrum to dorsal spine	Rostrum	Dorsal spine	Lateral spine	Carapace length	Abdominal length	Total length
I	1.75	0.40	0.85	0.26	0.64	1.37	2.01
	1.62–1.80	0.36–0.46	0.72–0.90	0.23–0.29	0.61–0.72	1.30–1.44	1.98–2.12
	24	25	24	19	25	25	25
II	1.94*	0.55	0.81*	0.27	0.85	1.55	2.41
	1.82–2.06	0.52–0.61	0.69–0.96	0.22–0.28	0.81–0.96	1.38–1.71	2.20–2.64
	15	28	15	30	30	30	30
III	2.38*	0.62	0.97*	0.26	1.17	1.75	2.93
	2.25–2.61	0.45–0.72	0.81–1.17	0.22–0.31	1.08–1.26	1.62–1.89	2.70–3.06
	19	30	19	27	28	30	28
IV	3.26	0.84	1.56	0.33	2.00	2.36	4.41
	3.03–3.44	0.83–0.88	1.24–1.68	0.28–0.36	1.73–2.20	2.20–2.61	4.04–4.68
	21	21	21	21	21	21	21
V	3.75	1.00	1.65	0.34	2.44	2.68	5.13
	3.58–3.99	0.86–1.03	1.51–1.79	0.28–0.41	2.26–2.53	2.48–2.75	4.95–5.28
	24	27	26	27	27	26	26

* Probably underestimated since the specimens measured were primarily from mass culture in which most had damaged dorsal spines resulting from attempted cannibalism.

Mxp 1 (Fig. 6a)—Coxa with 2 setae, basis with 10 setae on inner margin. Endopod with 2, 2, 1, 2, 6 setae on segments 1 to 5. Exopod with 12 terminal plumose setae.

Mxp 2 (Fig. 6b)—Coxa with 1 seta, basis with 4 setae on inner margin. Endopod with 1, 1, 5 setae, on segments 1 to 3. Exopod with 12 terminal plumose setae.

Mxp 3 (Fig. 6c)—Endopod with 5 pseudosegments, proximal (ischium) slightly broadened; 1 terminal seta. Exopod straight, nude. Epipod curved posteriorly into branchial chamber, nearly equals exopod, nude.

P 1 to 5—P 1 chelate, segmented; P 2 to 5 all segmented, pointed. Nearly fill central carapace space.

Pl 2 to 5 (Fig. 6d)—Biramous, lacking setae but with small knobs terminally.

U—Unchanged from Zoea IV.

It is clear from the degree of development observable in Mxp 3, P 1 to 5, Pl 2 to 5, and U, that this is the last zoeal stage, even though no megalopa was obtained. Thus like *Portunus, Bathynectes* has five zoeal stages.

Pigmentation: Black chromatophores were present in all zoeal stages on the basis of Mxp 1 and 2, the inner mouth parts, the labrum, ventrally on the cephalothorax, around the intestine, and posteroventrally on abdominal somites 3, 4, and 5. Eyes were dark brown. Abdominal somite 1 had a red-brown chromatophore. The telson was diffused with a pinkish hue. In Zoea IV, small yellow or white chromatophores were noted on the branchial and posterior regions of the carapace (very inconspicuous). They may have been present earlier, but were not noted. In Zoea V the chela of P 1 had a red-orange chromatophore as did the 6th abdominal somite, and in some cases the other pereiopods. At no time did the carapace bear any characteristic black chromatophores. Unlike Lebour's *B. longipes* Zoea I, larval *B. superba* did not have a chromatophore in the middle of the dorsal spine.

Mean dimensions of each zoeal stage along with the observed ranges and number of specimens measured are summarized in Table I. The zoeae increased from about 2 mm total length in Zoea I to about 4.4 mm in Zoea V. Growth increments were not linear but markedly saltatory with a very pronounced increase from Zoea III to Zoea IV of about 1.5 mm, all other growth increments being less than 1 mm. The same marked jump in growth increments was observed in every dimension measured. Examination of Lebour's (1928) body length data for *Portunus puber* shows the same phenomenon at the same place in the developmental sequence. No measurements were givn for *Callinectes sapidus* (Costlow and Bookhout, 1959), but a gross comparison of the figures of each stage suggests a similar jump in growth increment between Zoea IV and Zoea V, this latter species having 7 instead of 5 stages. This appears to be characteristic of this family, not having been reported in any other group.

Discussion

Lebour (1928) published a brief description and line drawing of the first zoea of *Bathynectes longipes* and later (1931) a colored figure. From this information there seems to be a distinction in pigment pattern between the larvae of *B. longipes* and *B. superba*. There is a distinct black chromatophore on the dorsal spine of *B. longipes* which is not present in *B. superba*. In addition, *B. longipes* seems to have more pigment overall than *B. superba*.

In external anatomy, the two species are more similar. A completely detailed comparison is not possible since *B. longipes* was not described in great detail nor are more than the first zoea known. Body length (total length less rostrum) of *B. longipes* Zoea I is about 0.5 mm less than *B. superba* Zoea I. In both species, the rostrum-dorsal spine length equals the body length. Carapace spines are in both cases strongly curved. The lateral spines are relatively long in both species, with a lateral spine: rostrum ratio of about 1:2 in *B. longipes*, *ca.* 3:5 in *B. superba*. This ratio is gradually reduced in *B. superba* to only 3:10 in Zoea IV. In Zoea I of both species, the A 2 exopod:peduncular spine ratio is *ca.* 1:2, endopod absent, peduncular spine nearly reaching rostral tip. The telson cannot be compared as it does not show clearly in the figures of *B. longipes.* There appear to be no posterolateral spines on abdominal somites 3, 4 and 5 in *B. longipes* while at least the first two of these spines are well developed in *B. superba* in Zoea I and all three in later instars.

Lebour (1928) indicated such great similarity between *Bathynectes* and *Portunus* as to preclude separation. Both have five zoeal stages; carapace with rostral, dorsal, and lateral spines; A 2 with truncated exopod, long peduncular spine, and endopod absent in early stages, developing in later stages; abdomen and telson similar. Aikawa (1937) claimed a difference in type of A 2 between *Bathynectes* and *Portunus* but this was most certainly unjustified both from Lebour's (1928, 1931, 1944, 1950) figures and consideration of *B. superba.*

There are differences between these two genera which, if they hold up as more species of *Portunus* become known, should permit separation. *Bathynectes* has a relatively longer lateral carapace spine than has *Portunus;* lateral spine:rostrum ratio equal to or greater than 1:2 in *Bathynectes* Zoea I, much less than 1:2 in *Portunus.* Even in later stages, there is probably a difference despite the reduction in this ratio in *Bathynectes.* The A 2 exopod:peduncular spine ratio is equal to 1:2 for *Bathynectes,* between 1:4 and 1:2 for *Portunus.* The posterolateral abdominal spines seem slightly better developed in *Bathynectes,* but this may result from inaccuracies in available *Portunus* descriptions. *Bathynectes* has 1 dorsal and 2 lateral spines on the telson furcae, one of the lateral spines (in *B. superba* at least) being very long. *Portunus* has only 2 lateral spines on the furcae of many species, 3 in some cases, but only in *P. spinimanus* (Lebour, 1950) is there a spine anywhere near as long as that of *Bathynectes.* As more larvae are described in better detail, additional differences will probably be detected.

Based on the description and figures of Costlow and Bookhout (1959), the same characteristics that distinguish *Bathynectes* and *Portunus* will also separate *Bathynectes* and *Callinectes. Callinectes* has seven zoeal stages compared to five for *Bathynectes* and *Portunus.* Therefore zoeae of *Callinectes* at comparable stages of development of pereiopod and pleopod buds will have more natatory setae on the Mxp exopods than the other two species. *Callinectes* has extremely short lateral spines, with a lateral spine:rostrum ratio of only 1:5 compared to 1:2 in *Bathynectes. Callinectes* has long posterolateral spines on abdominal somites 3, 4, and 5 in all stages, all three being about the same length, while they are all small in Zoea I of *Bathynectes* and the last one is smaller than the other two in all stages. The A 2 exopod of *Callinectes* is truncated with 2 unequal setae as in *Bathynectes* and *Portunus,* but the exopod:peduncular spine ratio is less than 1:4.

Bathynectes and *Carcinus maenas,* belonging to different subfamilies within the Portunidae, are more readily separated. *Carcinus* has only 4 zoeal stages before the megalopa. It lacks lateral carapace spines and postero-lateral spines on abdominal somites 3, 4, and 5. These differences have already been pointed out by Lebour (1928).

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SUMMARY

1. The external anatomy of 5 zoeal stages of *Bathynectes superba* is described from laboratory-reared specimens. Based on Mxp 3, P 1 to 5, Pl 2 to 5, and U structure in Zoea V, it is concluded that this is the last zoeal stage even though no megalopae were obtained.

2. It is suggested that *Bathynectes* can be distinguished from *Portunus* and *Callinectes* by differences in lateral carapace spine:rostrum ratio, A 2 exopod:peduncular spine ratio, spination of telson, and relative lengths of lateral abdominal spines.

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BODY CONSTRAINT AND DEVELOPMENTAL ARREST IN *GALLERIA MELLONELLA* L.: FURTHER STUDIES

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The sequence of events in insect development that follows activation of the prothoracic glands and culminates in molting or metamorphosis is now well established but the mechanism by which the sequence is initiated at the appropriate time and place remains little understood. It is reasonable to suppose that several or many requirements, such as nutritional state, location, and appropriate body form should be met before a larva molts, or enters the developmental crisis of pupation. If integrated information about these factors converges upon neurosecretory cells, either to activate them, or release them from inhibition, it should be possible to delay release of neuroscretory material, and thus suspend development. Until the neural pathways to the neurosecretory cells of the brain are worked out, evidence of the mechanism of release can only be circumstantial: it must be shown in each case that a manipulation which suspends development is not acting at a point in the sequence beyond prothoracotropic hormone release. The capacity of implanted endocrine organs, or injected hormone to override developmental arrest provides suggestive, but not conclusive information about mechanisms of arrest.

Treatments which may be expected to alter sensory input to the brain of *Galleria mellonella*, such as severing the ventral nerve cord or bodily constraint, prevent initiation of the larval-pupal molting cycle and it has been suggested that these operations may inhibit the release of prothoracotropic hormone as a result of modified proprioreceptive input (Edwards, 1967). Such treatments might in fact intervene in the molt controlling mechanisms at several levels: (a) by inhibiting the secretion of prothoracotropic hormone from the brain, (b) by blocking the response of the prothoracic glands to the hormone, or otherwise preventing the secretion of molting hormone, or (c) by directly affecting the capacity of epidermis to respond to the hormones.

The aim of this study was to limit these possibilities. It is shown that restraint neither blocks the capacity of the prothoracic glands to secrete ecdysone, nor the capacity of the epidermis to respond to ecdysone. The mechanism is thus limited to inhibition of brain activity. The precise means by which prothoracotropic hormone is withheld remains to be demonstrated, but the results are consistent with the hypothesis that modification of sensory input underlies the inhibition, and that least in the wax moth, body form is a significant factor.

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MATERIAL AND METHODS

Wax moth larvae of known age were used for all experiments. Larvae were grown on an artificial medium (Sehnal and Schneiderman, in preparation) at 30° C and 60–80% R.H. Larvae were restrained as previously described (Edwards, 1967) using Scotch brand "Magic Mending" tape No. 810. They were checked twice daily, and occasional escapees were again restrained. As in work reported earlier (Edwards, 1967) the adhesive tape was applied in such a way as to insure that heart beat and blood circulation persisted. The restraining tape did not act as a ligature. The larvae spun silk and excreted a dry paste of urate material. Up to three spiracles were occluded by adhesive tape, but oxygen deprivation does not appear to be significant in these experiments for local sealing of spiracles with wax does not suspend development. Longitudinal tracheal trunks evidently allow adequate ventilation.

The development of experimental animals was assessed by recording morphological changes in the integument and, after 10 days, by investigating the developmental stage of the internal organs (Sehnal, 1968). Development was also estimated in unrestrained larvae ligated behind the prothorax.

Sensitivity of restrained insects to the prothoracotropic hormone and to the molting hormone was checked using larvae of the 6th day of the last larval instar. In the first set of experiments, each larva received an implant of five brains taken from intact insects of the same age. Hosts were allowed to recover for five hours and were then restrained with adhesive tape. One group of larvae received combined brain, corpora cardiaca and corpora allata implants. Controls were implanted with thoracic ganglia or with pieces of gut.

In the second set of experiments, effects of molting hormone were estimated by injecting larvae either with alpha-ecdysone (Huber and Hoppe, 1965) or with ecdysterone (Hoffmeister, 1966). The first compound was synthesized by Syntex Co., California, the second was extracted from the roots of *Polypodium vulgare* (Jizba, Herout and Šorm, 1967). Each larva was injected with 8 μg of one of the substances dissolved in 4 μl of 10% alcohol, and then restrained with adhesive tape. Controls received equal amounts of the solvent alone. Sensitivity of non-restrained intact of ligated larvae was established in a similar way.

RESULTS

The effects of restraint and of ligature in relation to developmental stage are summarized in Table I. Except for larvae from well-formed cocoons (about one day prior to the larval-pupal ecdysis) all insects remained at the developmental stage reached at the time of restraint. No changes in either integument or internal organs were visible within 10 days of restraint, except for the emptying of the gut and occasional accumulation of uric acid granules in the Malpighian tubules. Starving intact larvae either pupated within 10 days, or died subsequently.

Ligature and restraint had similar effects on development. Only larvae from well-formed cocoons were able to pupate after isolating the brain and the prothoracic glands by ligation.

Implantation of extra brains into restrained larvae was without detectable effect in most cases (Table II). Few specimens initiated development and underwent

TABLE I

Effect of body restraint and of ligature on the development of Galleria *larvae*

Developmental stage	Days of the instar	Restraint		Ligature		Comments
		Total number	Number molted	Total number	Number molted	
	Penultimate larval instar					
Feeding larva	2	10	0	10	0	No developmental progress
	Last larval instar					
Feeding larva	3	15	0	30	0	No developmental progress
Preparation for spinning	5	15	0	30	0	No developmental progress
Onset of spinning	6	15	0	30	0	No developmental progress
Larva in well-formed cocoon	7	25	17	30	15	In affected insects internal organs as in pupae. Limbs, wings, and other organs wihch developed from imaginal discs are reduced in size and structure. Pupal cuticle not always sclerotized.

Controls left inteact but without food. Experiments evaluated 10 days after the respective treatment when majority of controls either died or molted.

a molt. Of those animals which did develop, insects with implanted brains molted into pupae whereas larvae with implanted complexes molted to forms intermediate between larva and pupa (Piepho, 1940). The molting process in the integument was not always completed and the new cuticle often remained untanned.

Unlike the implantations of brains, injections of alpha-ecdysone or ecdysterone were effective in inducing development in restrained animals (Table III). Diges-

TABLE II

Effect of implanted brains on restrained larvae

Developmental stage	Implant	Number of insects	Number molted	Comments
Larva at onset of spinning	5 brains	14	3	Molted into pupae (4 died)
Larva at onset of spinning	5 complexes brain-c. cardiaca-c. allata	14	4	Molted into intermediates between larva and pupa (2 died)

Experiments were evaluated 10 days after treatment. Within this period only one of 25 controls molted. Controls were implanted either with thoracic ganglia or with pieces of gut.

tion of the old cuticle by the molting fluid proceeded to various degrees in different individuals. New cuticle bore pupal characteristics; it was usually well-formed and occasionally tanned, but cell proliferation, which normally accompanies the molting process, was suppressed. Limbs and wings were consequently smaller and morphologically simpler than in normal pupae. Characteristic pupal structures, such as the mid-dorsal rib, were lacking or reduced. Furthermore, gaps developed in epidermis in prolegs and genitalia regions, where larval cells degenerated but new epidermis failed to proliferate from the surroundings.

Ecdysone had an identical effect on larvae ligated behind the prothorax, but in all cases injection of solvent alone did not cause development to proceed in restrained or ligatured larvae.

TABLE III

Effects of ecdysones on intact, ligated, and restrained larvae of Galleria

Developmental stage	Injected:	Total number of insects	Number molted	Number dead
		Intact larvae		
Larva at onset of spinning	8 μg of ecdysterone	10	8	2
		Ligated larvae		
Larva at onset of spinning	8 μg of ecdysterone	10	7	3
Larva at onset of spinning	8 μg of alfa-ecdysone	25	17	5
		Restrained larvae		
Larva at onset of spinning	8 μg of ecdysterone	15	11	4
Larva at onset of spinning	8 μg of alfa-ecdysone	15	10	1

Insects usually molted within 4 days after the injections; remaining larvae were discarded after 10 days. Injection of the solvent alone into the control larvae had no effect.

DISCUSSION

Insect development is regulated by the nervous and endocrine systems. The common scheme for the control of the molting process, which repeats several times in the course of insect ontogeny, involves three steps (reviews by Wigglesworth, 1964; Novak, 1966). (1) Various stimuli, such as light, moisture, temperature, and food, act, probably through nervous pathways, on the neurosecretory system of the brain. Under suitable conditions, which apparently include such internal factors as increase in body size and accomplishment of certain morphogenetic processes, prothoracotropic hormone (synonyms: activation or brain hormone) is released and activates the prothoracic glands. (2) Prothoracic glands in turn secrete the molting hormone, alpha-ecdysone or ecdysterone. (3) Molting hormone evokes the molting process.

Immobilization of *Galleria* by mechanical restraint as described above suspends development in both penultimate and last larval instars. Animals so treated may survive for long periods without detectable change other than slow depletion of metabolic reserves (Edwards, 1967). The developmental block cannot be due to starvation, because it occurs in larvae that have completed feeding in the last instar, and which have entered the "wandering" stage that leads to cocoon spinning. When feeding larvae are taken from their food they either molt, or die long before their restrained contemporaries die (Sehnal, 1966).

A critical point is passed after the cocoon formation has been completed beyond which restraint no longer suspends development. Larvae at this developmental stage also pupate after ligating off the brain and prothoracic glands. In the earlier period of the instar, when restraint is effective, presence of the brain and prothoracic glands is indispensible for development. This coincidence suggests that bodily constraint hampers the molting process by affecting these glands and thus interrupting hormone production.

Prothoracotropic hormone could theoretically be supplied to restrained larvae by implanting several extra brains. The results obtained with implanted brains were variable: since the molting process was initiated in some animals it is clear that the prothoracic glands remain sensitive to activation and capable of secreting ecdysone. It is well known that implantation of potentially active brains may have little effect on unrestrained larvae which are deprived of their own brains by head ligature (Kuhn and Piepho, 1936; Sehnal, unpublished observations). The variable behavior of isolated brains awaits explanation. It is beyond the scope of the present study, but it seems probable that deficiencies of titer and duration of secretion are involved.

When the action of both brain and prothoracic glands was circumvented by injecting larvae with alpha-ecdysone or ecdysterone, restrained larvae responded by molting. The resulting pupae were not perfect but the molt had all typical features: detachment of the old cuticle, its partial digestion, secretion of a new cuticle and its occasional tanning.

Since larvae which are deprived of brain and prothoracic glands by ligature also respond to ecdysones, the injected hormone evidently acts directly on the target tissues. Sensitivity of the targets does not appear to be affected by restraint; for non-restrained larvae, both intact and ligated, respond to ecdysones in the same way.

Our observations indicate that bodily constraint inhibits secretion by the brain. This supports the earlier assumption that appropriate sensory input from the periphery must precede secretion of the prothoracotropic hormone (Edwards, 1967). Mechanical restraint of the larva apparently modifies the input and thus prevents the whole molting process. The effect can be by-passed by providing the prothoracotropic hormone or molting hormone by injection. Our results show that restraint inhibits development at any time in the course of the last larval instar.

Hence, a secretion from the brain seems to be required for a prolonged period of time and not only for an instantaneous activation of prothoracic glands.

Several other examples in which proprioception has been implicated in the regulation of neuroendocrine events may be briefly noted. Wigglesworth (1934) concluded that distension of the body wall provided the signal for the initiation of the molting cycle in *Rhodnius*. Clarke and Langley (1963) postulated a role for proprioceptive monitoring of crop distension in growth and molting of *Locusta*, and a similar control system has been suggested in the regulation of digestive enzyme activity in adult tsetse flies (Langley, 1966). Finlayson (1967) has recently demonstrated that mechanoreceptor input plays a role in the initiation of puparium formation in larvae of *Glossina*. The onset and termination of successive phases of insect molting and maturation is regulated by surface contact and body form in a number of studies cited by Cottrell (1964) and in certain of these, proprioceptive monitoring of body form is implicit.

Further significant progress toward proof of proprioceptive function as a factor in regulation of certain neurosecretions must await work on the central processing of input from the various proprioceptors now known in insects (Finlayson, 1968), and on the neuronal connections with neurosecretory cells for which there now is neither neuroanatomical nor physiological data.

SUMMARY

Restraint of *Galleria* larvae by mechanical means suspends the capacity for development. Restraint inhibits, apparently by a nervous route, secretion of prothoracotropic hormone from the brain; prothoracic glands do not produce the molting hormone and larvae thus never molt. Targets of the molting hormone are not affected and respond normally to an artificial supply of the hormone. These results support the hypothesis that appropriate proprioceptive input is a necessary condition for the normal activation of the neuro-endocrine system.

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PHOTOPERIOD CONTROL OF DIAPAUSE IN *DAPHNIA*. III. TWO-STIMULUS CONTROL OF LONG-DAY, SHORT-DAY INDUCTION

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Females of *Daphnia*, as of all Cladocera, may be reproductively polymorphic, and produce two structurally and functionally different types of eggs, one of which later enters an embryonic diapause. Expression or display of the polymorphism has been claimed to be under the control of two stimuli (Stross and Hill, 1965; 1968). One of the stimuli is an inductively short photoperiod. The second is unidentified, but its effect may be proportional to density of culture. The indicated function of the second stimulus is to trigger expression when daylength is permissively short. The hypothesis of dual stimulation is further examined in this paper.

The validity of photoperiod, or daylength, control is supported by more recent study. Comparison of populations of *Daphnia* living at 45° and 71° N, although of different species shows a latitudinal adjustment in critical photoperiod. At 45° N it is 13 hours, and at 71° N it is 22 hours of light per day at constant temperature (12° C). Photoperiod has also been implicated in the display of the reproductive polymorphism of other species of Cladocera (Parker, 1966; Shan and Frey, 1968).

The question posed in the experiments reported here is whether or not photoperiod and a density-proportional stimulus may control expression of the reproductive polymorphism in all seasonal cycles. The hypothesis of dual stimulation is based on experiments with a monocyclic strain in which expression in nature is restricted to autumn. In *Daphnia pulex* Leydig there are at least two other seasonal cycles. In the acyclic strain, the adults produce only one kind of egg, and embryonic diapause is absent. In the dicyclic strain the reproductive polymorphism may be expressed during the long daylengths of late spring as well as in short daylengths of autumn. The apparent need for both long-day and short-day induction, which is seemingly necessary to explain control by photoperiod, presents a challenge to the hypothesis of dual stimulation since no single organism is known to be both long and short-day inductive for the same polymorphism.

An attractive feature of dual stimulus control is that the observation of early investigators may be explained. Berg (1931) places the early literature into three categories or viewpoints. One view, championed by Weismann (1879), believed control was intrinsic, except for certain labile periods, and the number of generations elapsing between successive expressions of the polymorphism was believed to be under genetic control. A second group believed control to result from a variety of external stimuli associated with culture density (*e.g.* metabolites, nutritional level, *etc.*) or temperature. A third viewpoint, held by Woltereck (1911) believed that the display resulted from an environmental induction of a pre-induced responsiveness of the organism. Woltereck (1911) identified the pre-inductive stages in the life cycle, and his embryonic stage of the mother corresponds to the most sensitive stage for induction by photoperiod (Stross and Hill, 1968). In retrospect it is possible to reinterpret intrinsic control as resulting from the then unrecognized action of daylength and extrinsic control to be the manifestation of what may be identified as the second or density-proportional stimulus.

With the recognition that photoperiod may be involved, some of the earlier descriptions of reproductive types of females (Grosvenor and Smith, 1913, Berg, 1931; 1934) have been abandoned, following Lees' (1959) treatment of the same phenomenon in an aphid. Females which are induced during embryonic or early post-natal life may produce only the short-day, or yolky, type of egg which enters an embryonic diapause. Females may also be induced later in life, and, as a result, may produce both types of eggs during their reproductive life. The short-day induced state is also reversible (Stross and Hill, 1968; Stross, 1969). Thus the distinction of female type, at least for present purposes, seems unnecessary. Also avoided in the description of a female type is a specific identification of females that produce broods of males. Males are a necessary part of the polymorphic display in some strains of *D. pulex,* although in other strains of the species and, presumably, in all strains of another species (*D. middendorffiana*) both types of embryos are produced parthenogenetically. In the bisexual strains, broods of males develop parthenogenetically and precede the shift to production of the yolky type of egg. In the one case examined by Stross and Hill (1968), the induction of males was also under the control of photoperiod, and a single female was found to be capable of producing young (both male and female) and the yolky eggs which later enter diapause. The significant feature of the reproductive polymorphism may be the shift from the production of broods of non-diapausing to the production of broods of diapausing embryos; such a designation facilitates description.

METHODS AND MATERIALS

Only minor modifications were imposed in the procedures reported earlier (Stross and Hill, 1968). The test animals, beginning as young, were cultured for 30 days under controlled photoperiods and temperatures at standardized densities. Experiments with both the dicylic and acyclic strains of *Daphnia pulex* were carried out with the descendents of one female.

Modifications in the procedures consisted of the use of filtered lake water (Rensselaer Lake, Albany, New York) after it had been allowed to stand in pyrex carboys from one to several weeks. Light intensity in the photoperiod chambers was 450 lux. Another modification was the maintenance of constant temperature in experiments carried out at 12° C during the bi-daily census and transfer to fresh food suspensions; transfer in experiments at 19° C was carried out at room temperature (21° to 24° C). All treatments are reported as the means of four replications and were tested for statistical significance when responses were intermediate to the all-or-none.

The dicyclic strain of *D. pulex* was collected from experimental ponds at Ithaca (42° N), New York. Adults were collected in June, and young were collected in November following termination of the summer period of diapause. Collections of Dr. Donald Hall, Cornell University, were examined to verify the late spring expression of the reproductive polymorphism. Collection during the winter confirmed the supposition that the population re-enters embryonic diapause in autumn.

The acyclic strain was collected from Lake Conesus (43° N), New York. This strain may be classed as operationally acyclic, since all adults in a collection made in early December 1968 were reproducing broods of the non-yolky, non-diapausing embryos. A collection (1 April, 1969) after the lake had been frozen for several months contained egg pods with the diapausing embryos, although the adults collected were producing broods of the non-yolky embryos only. The egg pod, or ephippium, in this strain lacks the characteristic black pigmentation, whether produced in the laboratory or lake.

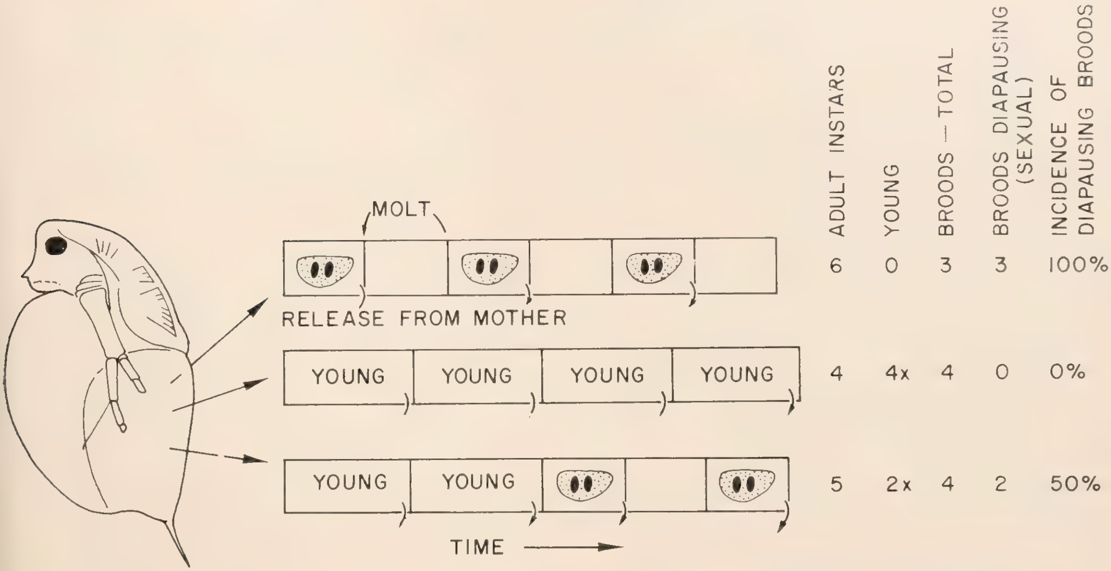

FIGURE 1. Sequence of reproductive instars in *Daphnia* and scoring procedure. The rationale for scoring broods rather than reproductive products (young and diapausing embryos) is diagrammed. See text for explanation.

Requirements for termination of the summer diapause were determined with embryos of *D. pulex* which had been collected from a small pond in Boulder, Colorado (40° N) in late August and transported to the laboratory while maintained in the natural light. In the laboratory one group was placed immediately into the experimental environments. A second group was divided, placed in sealed jars and stored in the dark at 4° or 20° C until May of the following year. The temperatures and photoperiods necessary to terminate diapause were tested in duplicates of 50 egg pods, containing 86.7 ± 4.4 (ideally 100) embryos, contained in beakers with 50 ml of lake water. The embryos, within the egg pods, were subjected to a light intensity of 1350 lux. The beakers were examined daily, after hatching began, those in dark less frequently with the aid of a safe lamp emitting far-red light.

The scoring procedure measures the output of broods yielding diapausing embryos relative to the total output of broods (Fig. 1). At two-day intervals all released young, egg pods, and exuviae are counted in each replicate. When more than one adult is present in a vial, the number of broods yielding young is determined by a combination of (1) the number of exuviae in excess of the number of egg pods, (2) the number of barren instars predicted by the previous release of diapausing broods, and (3) the probable number of young per brood. The uncertainty is small since any given adult usually produces only one type of brood throughout the assay period, and if reproduction shifts, it usually does so only once during the 30-day assay interval.

Ideally, one may wish to compare the time interval spent in producing young or diapausing embryos. The duration of an adult instar producing diapausing embryos may, at 12° C, be only $\frac{2}{3}$ as long as an instar yielding a brood of young (Fig. 1). Moreover, a barren instar normally follows an instar which produces diapausing embryos (Stross, 1969), such that total relative duration of successive broods of diapausing embryos is approximately 1.3 times the duration of successive broods of young; at temperatures above or below 12° the ratio may be different.

A procedure, often reported in the literature, is to add young and egg pods, or their embryos, but it is completely unsatisfactory. In well fed cultures the time devoted to producing one young may be only a fraction of that spent producing an embryo that enters diapause. For example, an individual female (or group of females) may spend similar amounts of time releasing broods of young and diapaused embryos, as illustrated in Figure 1. Scored as broods, the incidence of diapausing broods is $\frac{1}{2}$ or 50%. Scored as reproductive products in which the number of young (X) is say, 20 per brood, which often is the case in well fed cultures, the incidence of diapause is 1/21 or roughly 5%, a gross underestimate of the relative time devoted to producing diapausing embryos. The objective is, of course, to describe precisely the reproductive response of cultures exposed to daylengths intermediate to the inductive and non-inductive photoperiods.

Results

The dicylic strain

In contrast to the monocyclic, photoperiod control of the dicyclic strain is temperature sensitive within the range tested (13° and 19°). When cultured individually at 13° C, essentially all broods are of young at L16:D8 and all broods are of diapausing embryos at L12:D12 (Fig. 2A). The critical photoperiod, or 1:1 ratio of diapausing to non-diapausing broods, is approximately L13:D11, essentially the same as for the monocyclic strain which had been collected at a similar latitude (Stross and Hill, 1968).

Sensitivity of the dicyclic strain to photoperiodic induction is also maximal during embryonic and early postnatal life. Transferred to long daylength (L16:D8) from short daylength (L11:D13) 1 to 3 days after birth, 12% of the broods were of diapausing embryos (Fig. 2A). Born and tested in long days (L16:D8),

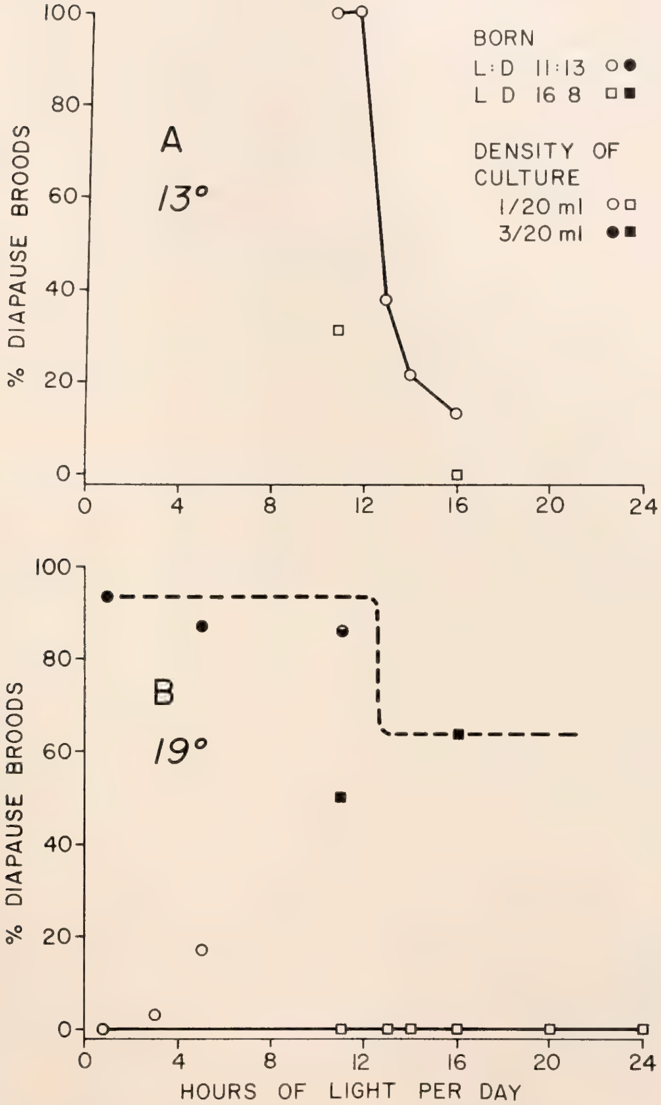

FIGURE 2. Environmental control of reproduction in the dicyclic strain. At 13° C (A) the reproductive switch to the release of diapausing embryos is short-day inductive. Mothers born in short days (open circles) are much more responsive to post-natal induction. At 19° C (B) the incidence of diapausing broods is nearly zero when the mothers are cultured individually (open symbols). When cultured at a density of 3 per 20 ml (solid symbols), the incidence is large, and larger when the mothers are born in short days (solid circles).

the adults produced only broods of young. Born in long days and tested in short, the adults produced only 31% ($P = 0.01$) diapausing broods as compared with 100% when born and tested in short days. A second test, also at 13° C, gave nearly identical values. The sharpness of the response to photoperiod when tested at a density of one adult per 20 ml and the restriction of maximum sensitivity to a limited phase of the life cycle are reasonable criteria of photoperiod control in the dicycle.

Display of the reproductive polymorphism at 19° C is primarily a function of culture density. There is essentially no photoperiodic effect when the dicyclic strain is cultured at a density of 1 per 20 ml at photoperiods ranging from L1:D23 to constant light (Fig. 2B). Only 2 of 36 females produced broods of diapausing embryos, and they were at L3:D21 and L5:D19. The apparent absence of a photoperiodic response when individual females are cultured at 19° C may be described as thermal uncoupling of photoperiod control. A similar response has been observed in insects (Paris and Jenner, 1959; Bunning and Joerrens, 1960; Lees, 1959), and could describe the results of a previous study with *Daphnia magna* (Mortimer, 1935).

The reproductive polymorphism may be displayed at 19°, however, and in long-day photoperiods. At 19° C, a water temperature comparable to that of late spring, display is controlled by a stimulus associated with density of the culture. Nearly all (93%) broods were of the diapausing embryos at a density of 3 per 20 ml, but only when the mothers were born in a short-day photoperiod (Fig. 2B). Born in a long daylength (L16:D8), and tested at the same density, the females produced a significantly smaller ($P = 0.01$) percentage of diapausing broods. The mean incidence of diapausing broods was 63.1%. The smaller proportion of diapausing broods released by mothers born in a long day-length may be the result of prenatal experience, since there was no statistical difference in the proportion of diapausing broods released in L16:D8 and in L11:D13 at 19° (Fig. 2B, solid squares). Additional tests verified the response. Therefore, it seems apparent that photoperiod continues to exert an effect at 19°, although its effect may be restricted to the prenatal or early post-natal life of the mother.

The requirement of dual stimulation was not demonstrated at 13° C, although a photoperiod and a density proportional stimulus may be functioning at 19° C. At 13° the response to short daylengths was the same (100% broods of diapausing embryos) at densities of one adult in 20, 50, and 100 ml. A density stimulus may exert an influence, however. For example, reversal of reproduction from the short to the long-day type is much slower when culture density is increased from 1 to 2 females per 20 ml.

The acyclic strain

Some of the populations free of embryonic diapause in nature may release broods of diapausing embryos in the laboratory (Berg, 1931). In this study the acyclic strain also displayed the reproductive polymorphism when cultured in the appropriate environment. At 13° C, which may represent autumn temperatures in the field, no diapause broods were produced in a long daylength (L16:D8; Fig. 3A). Culture densities were 1, 3 and 5 adults per 20 ml. In a short daylength (L11:D13), diapausing broods were produced, but only at densities of 5 and

10 adults per 20 ml (Fig. 3C). Since the total number of broods released per culture was, for practical purposes, constant at those two densities, the numbers of diapausing broods released per culture was subjected to an unpaired test of "t." The mean number released per culture at a density of 10 adults per 20 ml (10.8) was larger ($P = 0.01$) than the mean number of diapausing broods released at a density of 5 per 20 ml (2.0). The mean number of diapausing broods accounted for 73% of the total broods released at a density of 10 adults per 20 ml. Direct tests of the transformed (arc-sin) percentages are also significantly ($P = 0.01$) different.

At 19° C and short daylength, the incidence of diapausing broods was suppressed, as determined by comparison at a density of 10 adults per 20 ml (Fig. 3D). The number of diapausing broods was again larger ($P = 0.05$) at a density of 10 adults than at a density of 5 adults per 20 ml. The percentage of the total broods released as diapausing broods was 24.5% at L11:D13. There was a partial overriding of the long-day effect at the higher temperature but only at the maximum density tested, or 5 adults per 20 ml (Fig. 3B). There was also no difference in the reproductive response of adults whether born at the long or the short daylength.

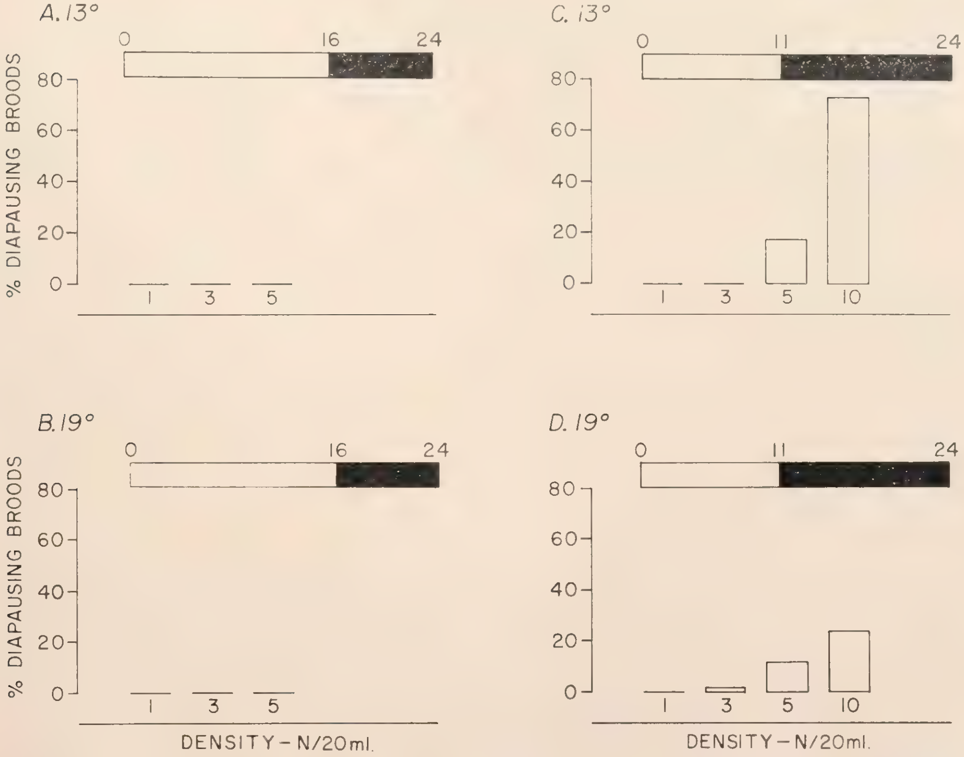

FIGURE 3. Display of the reproductive polymorphism in the acyclic strain in response to photoperiod and density. The reproductive shift occurs in short days and is transitional at a density of 5 adults per 20 ml. The shift is largest at the lower temperature.

The primary distinction of the acyclic strain seems to be its relative insensitivity to the crowding stimulus. Although individuals of the acyclic and dicyclic strains are equally large, a threshold density of 4 or 5 individuals per 20 ml is required to induce the reproductive shift in the acyclic strain. A single adult of the dicyclic strain is adequate for an essentially complete shift in reproduction when cultured in the same environmental conditions.

Male induction

In the monocyclic strain, males are short-day inductive (Stross and Hill, 1968). In the acyclic strain, males are also short-day inductive. The clone from Conesus Lake proved to be ideal for testing the response to daylength. The lack of a reproductive shift in short daylengths at low culture density permits continual observation of parthenogenesis, hence the conditions under which males are induced. At 13° C and short daylength, males were produced at all densities (1, 3, 5, and 10 adults per 20 ml; Table I). Males were produced with equal frequency to mothers born in either long or short days, and accounted for approximately 36% of the young released.

Temperature may be an important variable, both in this study and historically. At 19° C, the cultures yielded only an occasional male, or approximately 1.0% in both long and short-day photoperiods (Table I). A similar temperature influence has been observed on induction of males in the aphids (Kenten, 1955; Lees, 1959). Short daylengths acting through the food plants may also be required for induction of males in the aphid, *Megoura* (Von Dehn, 1967). Earlier reports of a temperature influence on the production of males are equivocal for *D. pulex* (Brown and Banta, 1932).

Broods of young *Daphnia* tend to be of a single sex as with another Cladoceran *Moina* (Banta, 1925). In cultures of the acyclic strain containing one adult, 13 broods were all females, 8 all males, and only 2 were of both sexes. In these cultures, the broods of males were released primarily in the second and to a lesser extent in the third brood. None of the first broods (8) contained males regardless of whether the mother was born at long or short daylengths. At densities of 3, 5 and 10 per 20 ml, males appeared in the first broods. The dicyclic strain did not produce males, nor are they required for the production of viable embryos.

Termination of summer diapause

Thermal and photic requirements for reactivation of embryos in summer diapause reveal a minimum of two-stages in a diapause development as observed in insects (Adkisson, 1965). The first or refractory phase was completed to some extent at 12° C (19.0% of viable embryos), maximally at 4° (80.0%) and not at all at 20° C. Summer diapause may have a thermal optimum slightly higher than observed for the winter diapause of the monocyclic strain. The partial effectiveness of 12° C in the former may be evidence of this. Masaki (1956) noted a higher thermal optimum in a summer diapausing moth. Some progress toward the completion of the refractory phase may occur at 20° C, although completion is unlikely. Embryos incubated for nine months at 20° C required only approxi-

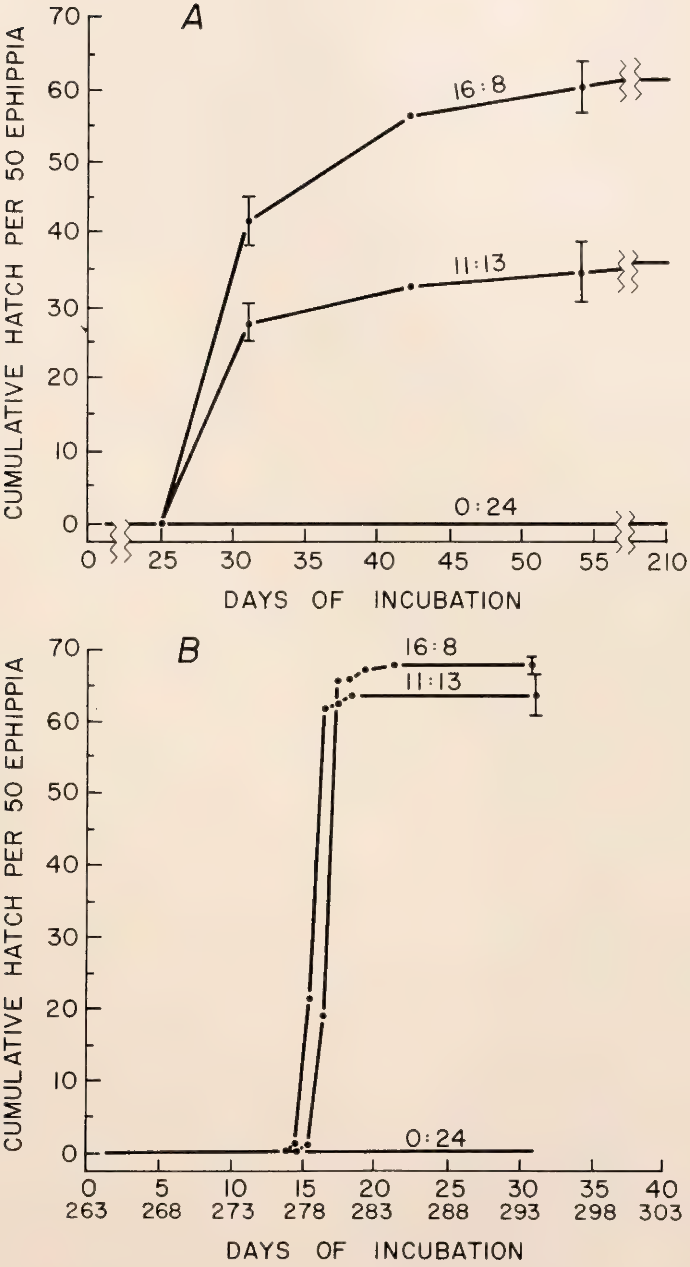

FIGURE 4. Hatching of diapaused embryos from a dicyclic strain of *D. pulex* in summer diapause. Embryos were incubated at 4° C in either the light or the dark. The influence of photoperiod (A) disappeared (B) after incubation at 4° in the dark.

mately one-half as much incubation at 4° C before becoming sensitive to the light stimulus.

The duration of the refractory interval may be only two or three weeks when incubated at 4° C. When transferred to 4° C four days after collection in August, the embryos began hatching sometime between days 25 and 30 of incubation (Fig. 4A). When the embryos were incubated for some months in the dark at the same temperature, and then exposed to light, hatching began on day 15 following transfer of the embryos to light. The 15-day interval may be essentially all post-diapause development at 4° C. Subtracting 15 days from the median time of 29 days required for hatching immediately after collection may suggest the time actually required. The two weeks is approximately one-tenth the duration of the refractory interval of winter diapause in the monocyclic strain (Stross and Hill, 1968).

Table I

Photoperiod, density, and temperature and the production of males in a clone from an acyclic strain of Daphnia pulex. *Mothers were cultured for a period of 30 days, beginning as young as 0 to 2 days old.*

		Density (Number/20 ml)				
		1	3	5	10	Totals and means
Test temp. 13°C						
Test photoperiod 16:8						
Parents born 11:13	young	80	186	256	—	522
	% males	0.0	0.0	0.0	—	0.0
Parents born 16:8	young	110	154	189	—	453
	% males	0.0	0.0	0.0	—	0.0
Test photoperiod 11:13						
Parents born 11:13	young	128	187	166	—	481
	% males	46.1	29.9	39.7	—	36.0
Parents born 16:8	young	121	162	150	39	472
	% males	43.8	26.5	38.0	41.0	35.8
Test temp. 19°C						
Test photoperiod*						
Parents born 18:8	young	377	689	571	—	1637
	% males	2.4	0.6	0.0	—	0.8
Parents born 11:13	young	346	538	436	372†	1692
	% males	0.0	1.3	0.0	1.6	0.8

* Parents born at both L16:D8 and L11:D13.

† Parents born only at long days (L16:D8).

The brief duration of the refractory phase of diapause development is evidence that a dicyclic strain may function in nature. That is, the same population may be active in spring and in autumn after spending the summer in an embryonic diapause. It should be stressed that the above test of the refractory interval may be applicable only to diapaused embryos which have been exposed to a summer environment for an unknown interval prior to collection.

A light stimulus is required for completion of the second, or photo-sensitive phase of diapause. Moreover, the light could be demanded in the form of a long-day photoperiod. Only embryos incubated in light were activated (Fig. 4A). Also, the number of embryos which hatched in a long daylength (L16:D8) was significantly larger than the number in a short daylength (L11:D13). The fact that embryos hatched in the short photoperiod may be interpreted either as the absence of photoperiod control in a part of the population, or the interaction of an uncontrolled variable with the photoperiod control mechanism. The latter is suspected. The absolute requirement for light persisted while the photoperiod effect disappeared after incubation at 4° C in the dark for nine months (Fig. 4B). Embryos of the monocyclic strain may lose the absolute requirement for light during the course of diapause development (Stross and Hill, 1968).

Discussion and Conclusions

Three strains of *Daphnia pulex* may display a reproductive polymorphism in response to two stimuli. Each strain was selected to represent one of three seasonal patterns identified by Berg (1931). All three strains may respond to photoperiod. They seem to differ primarily in sensitivity to the second, or density proportional, stimulus. Comparison of the acyclic and dicyclic strains suggests a several-fold difference in sensitivity. Two of the strains, the monocyclic and the dicyclic, may also differ in the temperature at which photoperiod control becomes uncoupled. In this study, what is described as thermal uncoupling was evident in the dicycle strain at 19°, while in the monocyclic strain there was no evidence of it at 19° C (Stross and Hill, 1968).

The requirement for two stimuli is clearly revealed in the reproductive pattern of the dicyclic strain. At autumn temperatures (13° C and colder) the production of diapausing embryos is short-day inductive. At late spring temperatures (19° and warmer), photoperiod is not completely effective and the reproductive shift is effected by a stimulus whose effect may be density-proportional. The less extensive shift of mothers born in a long daylength at 19° C is taken as evidence that photoperiod continues to exert a partial effect at that temperature. In other words thermal uncoupling, or a loss of temperature compensation of a photoperiodic process, may permit the reproductive shift in long daylengths when the density stimulus is present. The breakdown of temperature compensation is often observed in arthropod photoperiodism (*e.g.*, Bunning and Joerrens, 1960; Paris and Jenner, 1959). Presumably the dicyclic strain may differ from the monocyclic strain (reproductive shift in autumn only) in that some mechanism, under the control of photoperiod, becomes uncoupled at a lower temperature. Although untested, the two strains could also differ in their sensitivity to the density stimulus.

Support for a simultaneous requirement of inductive photoperiod and a density-proportional stimulus is supplied by the response of the acyclic strain at 13° C. Although releasing only young in nature during the short daylengths of late autumn, the acyclic strain may be induced to release diapausing embryos in the laboratory in short-day photoperiods, but only when sufficiently crowded. The acyclic strain is apparently less sensitive to the density stimulus than is the monocyclic and discyclic strains, and its higher threshold of sensitivity may distinguish it as Berg (1931) has concluded.

Photoperiod control of male induction was shown earlier for the monocyclic (Stross and Hill, 1968) and in this paper for the acyclic strain. In the monocyclic strain, it was found that only mothers exposed after birth to inductive photoperiods actually produced males. Embryonic induction always resulted in the production of diapausing embryos. In the acyclic strain males were produced by mothers exposed both before and after birth to an inductive photoperiod. However, since young, hence males, may not be produced in the monocycle when the mother is induced prenatally, there is no basis for comparing the two strains.

Earlier work reviewed by Banta (1939) suggested separate stimuli were responsible for inducing males and the reproductive shift, the former by an excretory product in the medium (Banta and Brown, 1929a, b) and the latter by food deprivation, although direct support for separate stimuli may be lacking. Evidence for distinct stimuli is equivocal in the present work. Adults, presumably well fed, which release broods of 15 or more young in long daylengths may be induced to release only diapausing embryos in short daylengths. Thus, food level seems to be ruled out. However, it may be argued that a density stimulus may not be required in the situation, *e.g.*, dicyclic strain at 13° C, where a single individual is sufficient to display the polymorphism. It may also be argued that photoperiod also acts indirectly through the food supply, as in male induction of the aphid, *Megoura* (Von Dehn, 1967). This seems possible, although unlikely, since the alga is cultured in a long daylength and spends a maximum of only 48 hours in the culture vessel, although usually less since most of the cells may be consumed in a time period shorter than 48 hours.

In any event there can be no argument as to the identity of the density stimulus in the situations where the intensity of display of the polymorphism is a function of density. No experiments were undertaken to distinguish between nutritional level and metabolites. Identity of the stimulus, or stimuli, remains to be determined.

Apart from the identity of the stimulus, an important question may be whether the density stimulus functions as an independent, or as an accessory stimulus to photoperiod. There is evidence to support the latter alternative. Models of photoperiod control (Bunning, 1959; Lees, 1966; Pittendrigh, 1966) agree that a photo-inducible phase of a natural rhythm lies somewhere near the beginning, or end, of the dark period of the environment; short-day induction is achieved when the inducible phase of the organism lies within the dark period of the environment. In one model (Pittendrigh, 1966) the photo-inducible phase is proposed to be coupled to the circadian oscillation. However, for the sake of illustration, one may propose that the photo-inducible phase in *Daphnia* is not coupled, and, furthermore has a natural tendency to float in the photophase of the environmental cycle, regardless of the length. Now, it is suggested that a function of the density stimulus could be either to displace a photo-inducible phase into the dark period or to couple it to a specific phase of the circadian oscillation. Evidence for both is circumstantial although from a variety of sources.

Evidence for phase deplacement is seen in the shift of the critical photoperiod to a longer daylength when an arctic strain is grown in dense cultures. An increase in density was shown to shift the critical photoperiod by more than one hour (Stross, 1969). The shift is interpreted as a small but real displacement of a photo-inducible phase.

Larger shifts are suggested in the process of male induction. The vast literature describing male induction in the Cladocera, identified above as a photoperiodically controlled process, abounds with evidence of phase displacement as a result of culture density. The phenomenon of male induction may be ideally suited since males are produced in adult instars whose beginning and end could occur at no specific time relative to the light-dark cycle. (In *Moina*, for example, the first ovulation is 65 hours after birth at 20°, 38 hours at 25° C (Banta and Brown, 1929c), and presumably intermediate at intervening temperatures (Brown, 1926). In *Daphnia* the average duration of adult instars releasing young includes fractions of a day (Stross, 1969) and ovulation is fixed to molting. The second essential fact is that at minimum density, the first brood contains only females in *Moina* (Banta, 1925) and *Daphnia* (see above), while second and/or third broods may contain males. An increase in culture density results in the appearance of males in the first brood, a fact which formed the basis of the assay in later studies by Banta. That crowding may have acted by causing a phase displacement is revealed in experiments by Banta and collaborators.

Banta and Brown (1929c) identified, through manipulation, a critical time for male induction at approximately four hours prior to ovulation. Later studies (Banta and Stuart, 1932) found that with other conditions the inductive period occurred 12 to 14 hours before ovulation. Woltereck (1911) identified a similar critical period in *Daphnia* and a second period which occurred much earlier in the development of the mother; the second or earlier period is consistent with photoperiodic induction.

The action of the crowding stimulus was to effect a delay in development and release of broods. When nearly all of the first broods released by a culture were males, the delay was approximately 13 to 14 hours (Banta and Brown, 1929d). The actual development of male broods was reported to be only ½ hour longer. Thus the delay comes during the period prior to the release of ova into the brood pouch of the mother. Although inconclusive, it is reasonable to suspect that a density stimulus had displaced a photo-sensitive phase into the dark period.

The possibility of phase displacement is also suggested by the interaction of the density stimulus and temperature. The appearance of males in first broods of *Moina* is both a function of density and temperature (Brown and Banta, 1932). In the range of 14 and 21° C, the proportion of male broods was proportional to density. Above 21° and below 30° C an increase in culture density decreased the proportion of male broods. At 30° C an increase in culture density was again effective. In other words, crowding could be effective only when the critical period for sex-determination may be shifted to occur in the dark period of the environment, and conceivably this happens when a supposed ovarian cycle is entrainably circadian (14 to 21° C) and when it could be semi-circadian (30°). The density stimulus could act through endocrine mediation similar to that visualized by Lees (1963) or directly on the phasing of oogenesis in the target organ (ovary).

While unidentified, the density stimulus could prove to be a non-stress or token stimulus. Banta and collaborators showed that it is proportional to culture volume; one animal in 7.5 ml gave a response similar to 10 animals in 75.0 ml of medium. It is apparently non-specific and a variety of aquatic invertebrates may supply the stimulus as indeed would be the case if the stimulus is as suggested by Loomis (1957, 1964). The delicacy of stimulus action may be exemplified by an experiment of Stuart, Cooper and Coady (1933) in which the simple stoppering of the culture vessel virtually suppressed the inductive effect of the density factor on sex determination while not affecting the number of young produced.

The well known "token" effect of the photoperiod stimulus permits the photoperiodically sensitive segment of a population to develop to the diapause (resistant) stage in a non-stressing environment. The life cycle of a terrestrial arthropod may thereby be phased to a periodically harsh physical environment. An aquatic species, *Daphnia pulex* may be capable of coordinating its life cycle in response to stimuli from both its physical (photoperiod) and its biological (density-proportional stimulus) environments. Indeed the biologically derived stimulus seems capable, in different strains, of overriding and mediating the stimulus from the physical environment. Since the biological stimulus may supply the key trigger, it is reasonable to suppose that it too functions as a cue, or token stimulus. It is generally accepted that population abundance and its competitive ability with ecologically related species may depend on the size of the initial population each spring. Initial density is obviously a function of the density of diapaused individuals and the degree of synchrony in diapause termination. To suppose that "unfavorable external conditions" (Berg, 1934), or stress inducing stimuli, are cause for the reproductive shift in *Daphnia* would seem to deny a basic strategy of arthropod populations, namely, to maximize the output of diapausing individuals.

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SUMMARY

The reproductive polymorphism in *Daphnia pulex* may be under the control of photoperiod and a second stimulus whose effect is proportional to culture density. Originally tested with a monocyclic strain, the hypothesis is extended to describe control in two other strains of *D. pulex*.

The dicyclic strain which displays the polymorphism in both long and short-day photoperiods may be only short-day inductive by usual standards. Long-day expression results when photoperiod control is thermally uncoupled. Display of the polymorphism is then under the control of culture density.

The acyclic strain, which in nature omits the reproductive polymorphism, may be induced in the laboratory. It is the acyclic strain which demonstrates the simultaneous requirement of inductively short photoperiod and the density proportional stimulus. The three types of seasonal cycles in *D. pulex* are presumed to be genetically distinct responses to the two control stimuli. The dicyclic strain may be the most sensitive to thermal uncoupling of photoperiod control, and the acyclic strain may be least sensitive to the density stimulus.

An embryonic diapause results from display of the polymorphism. The dicyclic strain may function by virtue of a much shorter refractory interval which, for the summer diapause, was found to be approximately two weeks at 4° C.

Light is an absolute requirement for termination of the diapause, and it is suggested to be more effective when presented as a long-day photoperiod.

In an attempt to relate the requirement for dual stimulation, the reproductive polymorphism of *Daphnia* is suggested to belong to a class of polymorphisms which requires external assistance in the temporal positioning of a photo-inducible phase. Expression of the polymorphism is presumed to occur when a photo-inducible phase is displaced into the dark period of the environment by a second stimulus. Several lines of evidence, which are taken from earlier studies of sex induction in the Cladocera, seem to support the idea.

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DIFFERENTIAL RADIOPROTECTION BY GLUTATHIONE OF TWO GROWTH FUNCTIONS IN THE HYDROID *CAMPANULARIA FLEXUOSA*

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The phenomena of growth, development, and regeneration in cnidaria have been previously studied by the use of x-irradiation. In most cases, x-irradiation leads to deleterious changes in these organisms. Puckett (1935) reported that the regeneration of amputated hydranths of the hydroid *Pennaria tiarella* was prevented by an x-ray dose of 10,000 roentgens. Daniel and Park (1951, 1953) produced considerable damage to the tentacles of the common brown hydra either by x-irradiating the animals with 9600 roentgens, or by incubating nonirradiated animals in irradiated saline solution. Damage was considerably reduced if the animals were irradiated in 10^{-5} *M* glutathione-saline solution, or if 10^{-5} *M* glutathione was added to the saline solution before the solution was irradiated, and before nonirradiated animals were added to it.

On the other hand, Brock and Strehler (1963), and Strehler (1964), employing x-ray doses of from 500 to 210,000 roentgens on ten-day-old colonies of *Campanularia flexuosa*, reported an increase with increasing dose in the life span of the normally cyclically regressing hydranths of this species. Wermuth and Barnes (1967) in a preliminary report presented evidence for an increase in the growth rate of new, nonirradiated stolons of *Campanularia flexuosa* when the starting material of the new colonies received an x-ray dose of 81,000 roentgens on the fifth day after the initiation of the colonies. The present study extends these observations to include a study of the effects of glutathione incubation on the previously reported increase in new stolon growth rate. New hydranth production under conditions of irradiation, and the effects of glutathione incubation followed by x-irradiation were also observed. Finally a hypothesis concerning stolon growth control is proposed.

MATERIALS AND METHODS

Stock colonies were originally obtained from Dr. P. Sears Crowell, and Mr. Robert Suddith, Department of Zoology, Indiana University, Bloomington, who had collected them near Woods Hole, Massachusetts. The stock colony organisms have been vegetatively propagated as a clone for the past two years. Cultures of test organisms were established according to the method of Crowell (1953). The starting material for a new colony, consisting of a hydranth-bearing stem (upright) from a stock colony, was slipped under a cotton thread previously tied around a microscope slide. Within a day, the cut end of the upright repaired itself and new stolon growth began from the cut end. The term "starter" designates the upright and associated hydranths originally placed under the cotton thread; the term "stolon" designates the minimal amount of the starter upright on the side of the thread opposite the starter, and the new material which grows out from the cut end (Fig. 1).

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The slides with attached colonies were placed in glass staining racks. The colonies of the first four series were placed in glass staining dishes on a moving table. The sea water in the staining dishes was changed weekly. All subsequent colonies were placed in a flowing sea-water system. The temperature of all sets of colonies was maintained at 12° C. Colonies were fed once a day on *Artemia* (brine shrimp) nauplii just prior to examination. Each feeding hydranth was allowed to capture as many brine shrimp as possible. X-irradiation was delivered by a General Electric Maxitron Two-Fifty x-ray machine operated at 250 KVP and 15 milliamperes unfiltered. The distance from the x-ray source to the test colonies was 15 cm. Colonies to be irradiated were placed on a lead plate in an open Petri dish and then just covered with sea water. This Petri dish was placed on a larger, covered Petri dish filled with crushed ice. For differential irradiation, three one-eighth-inch thick lead plates were placed over the part of the colony to be protected. X-ray dosage as measured by a Victoreen r-meter at the position of the animals was 81,000 roentgens (8100 r/min for series 1 through 8, and 7600 r/min for series 9 through 18). Stray irradiation under the shielding was 40–50 r/min. Either whole colonies, starters only, or new stolons only were irradiated.

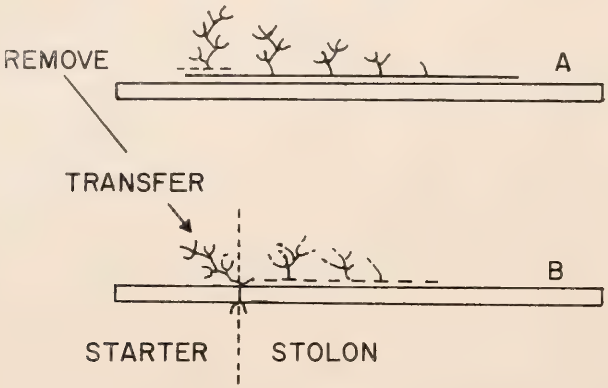

FIGURE 1. The method of starting new colonies of *Campanularia flexuosa* (after Crowell, 1953); A. Stock colony, B. new colony.

In those experiments involving glutathione incubation before irradiation, all colonies were placed in 10^{-5} *M* glutathione-sea water solutions in glass staining dishes. Glutathione-incubated colonies were irradiated in fresh sea water, except for the colonies of series 17. The pattern of irradiation, and glutathione incubation and subsequent irradiation for all the colonies described in this paper are listed in Table I. The glutathione used in these experiments was obtained from Calbiochem (lot number 73398) as crystalline reduced glutathione.

The standard "t" test, as described by Edwards (1958) was used to test for the possible statistical significance of the difference between the average daily stolon lengths of paired series.

RESULTS

Irradiation of growing stolons with 81,000 roentgens produces the following changes in the stolon. Three days after irradiation, the new stolon stops growing. By the fourth day, the normally bulbous-appearing stolon tip loses its rounded appearance and becomes square. Half-moon-shaped "dents" begin to appear in the sides (and presumably the top and bottom) of the coenosarc of the irradiated stolon. Eventually the dents enlarge so that the coenosarc is discontinuous. Within a week to ten days after the cessation of stolon growth, all cellular material has disappeared, presumably by autolytic processes, leaving the empty perisarc tubes undisturbed. In stolon-only irradiated colonies, new stolons may push into these hollow perisarc tubes from non-irradiated starters.

TABLE I

Pattern of irradiation and glutathione incubation followed by irradiation

Series	Number of colonies	Part of colony irradiated, and day of irradiation	Glutathione incubation
1	7	None	None
2	9	Stolon only, day 5	None
3	8	Starter only, day 5	None
4	7	Whole colony, day 5	None
5	9	None	All colonies of these four series were incubated in a freshly prepared solution from day 4 to day 5.
6	9	Stolon only, day 5	
7	9	Starter only, day 5	
8	8	Whole colony, day 5	
9	6	None	None
10	10	Starter only, day 6	None
11	8	None	Prep. day 4, inc. days 4–6
12	9	Starter only, day 6	Prep. day 4, inc. days 4–6
13	9	None	Prep. day 3, inc. days 4–6
14	9	Starter only, day 6	Prep. day 3, inc. days 4–6
15	11	None	Prep. day 4, inc. 30 min day 4
16	11	Starter only, day 6	Prep. day 4, inc. 30 min day 4
17	11	None	Prep. day 6, inc. during irr.
18	9	Starter only, day 6	Prep. day 6, inc. during irr.

Irradiated uprights are unable to produce new hydranths. The hydranths of this species undergo a regression-regeneration cycle (Crowell, 1953). Beyond a certain developmental stage, an irradiated hydranth will continue to differentiate until it becomes a complete, normal feeding structure. An irradiated complete

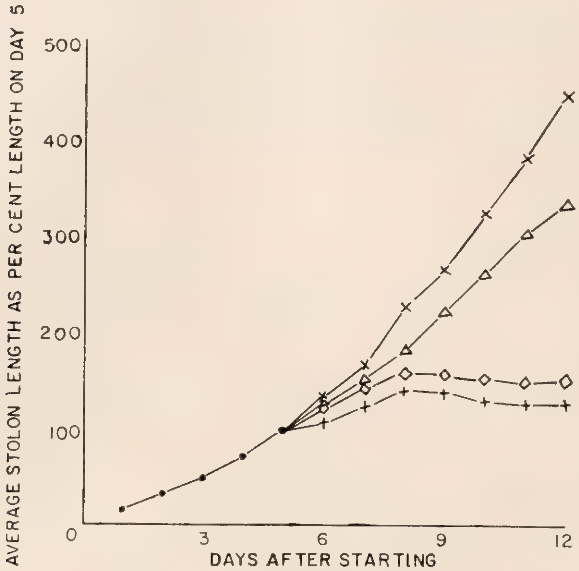

FIGURE 2. Average daily stolon lengths of colonies of *Campanularia flexuosa* as per cent of length on day irradiated (day 5) *vs* time. Colonies were not incubated in glutathione-sea water before irradiation. Symbols are: △, no irradiation (series 1); +, stolons only irradiated (series 2); X, starters only irradiated (series 3); ◇, whole colonies irradiated (series 4); ●, average of series 1 through 4 from day 1 through day 5.

hydranth will not regenerate after regressing, nor will an irradiated regressing or regressed hydranth regenerate a new hydranth. These irradiation-caused changes in stolons and hydranths are not reversed by incubation in glutathione-sea water solutions.

The results of x-irradiation on new stolon growth after the irradiation of new stolons only (series 2), starters only (series 3), and whole colonies (series 4) are presented graphically in Figure 2. The new stolons of starter-irradiated

TABLE II

Effects of x-irradiation, and glutathione incubation followed by x-irradiation on new stolon growth. Results of "t" tests (as "P" values)* for differences between average daily stolon lengths of paired series. For experimental situation of each series, see Table I.

Series	Day						
	1–5	6	7	8	9	10	11
1 and 3	—	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
2 and 4	—	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
5 and 7	—	<0.01	<0.02	<0.05	—	—	—
6 and 8	—	—	—	—	—	—	—

* (—) represents values of $P>0.05$.

colonies (series 3) continue to grow after irradiation. Furthermore, they grow at a greater rate post-irradiation than do new stolons of control colonies (series 1). The new stolons of wholly irradiated colonies (series 4) achieve a greater rate of stolon growth than do the stolons of stolon-only irradiated colonies (series 2), at least for the first three days after irradiation. At this time, the irradiated stolons of the stolon-only irradiated colonies of series 2 and the irradiated stolons of the wholly irradiated colonies of series 4 stopped growing. Probability values for the significance of the differences between the average daily stolon lengths of series 1 and series 3, and series 2 and series 4 are presented in Table II. For days 6 through 11, the differences between the average daily values of stolon lengths are highly significant ($P < 0.01$) for series 1 and 3, and series 2 and 4.

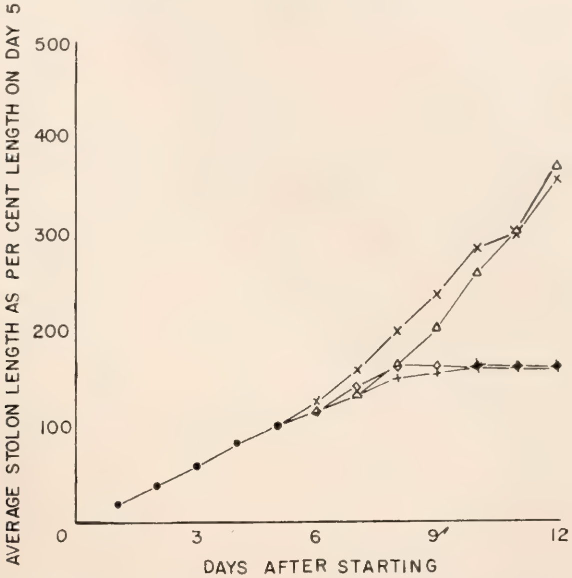

FIGURE 3. Average daily stolon lengths of colonies of *Campanularia flexuosa* as per cent of length on day irradiated (day 5) *vs* time. Colonies were incubated in fresh glutathione-sea water for 24 hours before irradiation. Symbols are: △, no irradiation (series 5); +, stolons only irradiated (series 6); X, starters only irradiated (series 7); ◇, whole colonies irradiated (series 8); ●, average of series 5 through 8 from day 1 through day 5.

The studies by Daniel and Park (1951, 1953) led us to test whether reduced glutathione in sea water would alter the radiation-induced changes in stolon growth and hydranth production described above. Accordingly, another four series of colonies (series 5 through 8) were irradiated in fresh sea water after they had been incubated in a freshly prepared 10^{-5} *M* reduced glutathione-sea water solution. A list of these experiments appears in Table I.

The results of the effects of x-irradiation at day 5 of new stolon growth of colonies which had been incubated in a freshly prepared glutathione-sea water solution from day 4 to day 5 are presented in Figure 3 and Table II. While there are statistically significant differences between the average daily values of stolon lengths of series 5 (no irradiation) and series 7 (starters irradiated) for the first three days after irradiation, this significance is not shown for the differences on days 4 through 6 after irradiation. There are no statistically significant differences between the average stolon lengths of series 6 (stolons only irradiated) and series 8 (whole colonies irradiated).

A 10^{-4} M solution of reduced glutathione in sea water was analyzed for changes in concentration according to the method of Jocelyn (1967). The concentration of reduced glutathione was found to decrease to one-half of its original value in approximately 30 minutes. We therefore designed a pattern of glutathione incubations followed by irradiation to determine whether reduced glutathione *per se* had a radioprotective effect. These experiments (series 9 through 18) were limited to starter-irradiated and nonirradiated colonies. Irradiation was applied on day 6 after the initiation of new colonies. A list of these experiments appears in Table I.

TABLE III

Effects of glutathione incubation followed by x-irradiation on new stolon growth. Results of "t" tests (as 'P' values)* for differences between average daily stolon lengths of paired series. For experimental situation of each series see Table I.

Series	Day						
	1–6	7	8	9	10	11	12
9 and 10	—	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
11 and 12	—	—	—	—	—	—	—
13 and 14	—	—	—	—	—	—	—
15 and 16	—	—	—	—	—	—	—
17 and 18	—	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

* (—) represents values of $P > 0.05$.

The results of the effects of x-irradiation applied to starters on new stolon growth after variable incubation in glutathione-sea water solutions are presented in Table III. The relative rate of new stolon growth of the nonincubated starter-irradiated colonies (series 10) is still significantly greater than the relative rate of new stolon growth of the nonincubated nonirradiated control (series 9). The pattern of these results is the same as that for series 1 and 3. In the remaining series of this set (series 11 through 18), statistically significant differences for average daily stolon lengths were found only for the paired series 17 and 18 (Table III). The colonies of these two series were incubated in a freshly prepared 10^{-4} M reduced glutathione-sea water solution during the course of irradiation on day 6 (series 18), or were incubated in glutathione-sea water for 10 min 40 sec on day 6 (series 17). The differences in average daily stolon growth values on nonirradiated and starter-irradiated colonies were statistically nonsignificant when colonies were incubated in fresh glutathione-sea water for 48 hours before irradiation (series 11 and 12); when colonies were incubated in 24-hour-old glutathione-sea water for 48 hours before irradiation (series 13 and 14); or when colonies were incubated for 30 minutes on day 4 in fresh glutathione-sea water.

Discussion

It would appear from the results of series 1 through 4, and series 9 and 10 that the rate of growth of new stolons of young colonies of *Campanularia flexuosa* is at least partially controlled by some part, or all of the upright used to initiate the colonies. The control seems to be of an inhibitory nature. Under certain conditions of irradiation of the starter upright, this inhibition of the rate of new stolon growth is suppressed resulting in an increased rate of new stolon growth. The evidence for the role of inhibition in the growth and regeneration of cnidarians has been well documented by Morgan (1901), Lund (1921), Rose (1957), and others.

The increased rate of stolon growth of starter-irradiated colonies disappears under certain conditions of incubation in 10^{-5} *M* reduced glutathione in sea water (Fig. 3, Table II and III). In these cases the differences in the relative rates of stolon growth of starter-irradiated colonies and their nonirradiated counterparts are not statistically significant. We interpret these experimental results as the radioprotection by some form of glutathione of an inhibitor system controlling stolon growth. An alternate hypothesis is that of radiosensitization by some form of glutathione. If the radiosensitization hypothesis is correct, one would expect the maximum average values of new stolon length achieved by the starter-irradiated colonies which have been glutathione incubated (series 12, 14, 16, and 18) to exceed the maximum average value of new stolon length achieved by the non-incubated starter-irradiated series (series 10). This is not the case.

It would seem that reduced glutathione is not the radioprotective substance, but rather that either some reaction product of reduced glutathione in sea water, or some metabolic product of glutathione is the radioprotective substance. This conclusion is derived from the following observations and assumptions. When colonies were incubated in a freshly prepared solution of reduced glutathione in sea water for 48 hours prior to irradiation (series 11 and 12), the concentration of reduced glutathione in the incubating medium would have been lowered to nearly zero by the end of the incubation period, assuming that the concentration of reduced glutathione decreases by one half in each half hour period. For series 13 and 14, "aging" the reduced glutathione in sea water lowers the concentration to about 5000 molecules per 250 ml of solution at the time the colonies were added to the solution for incubation. Considering the volume of the tissue of the colonies, and assuming that the reduced glutathione diffuses readily into the tissues of the colonies, it would seem that there was insufficient amounts of reduced glutathione present in the tissues to be radioprotective. Incubation of colonies for 30 minutes, 2 days prior to irradiation (series 15 and 16), and the resulting radioprotection afforded the proposed inhibitory system indicates that 30 minutes is sufficient time for the reduced glutathione or its sea-water reaction product to enter the tissues of the colonies. The 48 hour time lag between the end of the incubation period and the irradiation of the starters of the colonies suggests that some metabolic product of reduced glutathione is the radioprotective substance. If the preceding analysis is correct, it is not surprising that incubation in the reduced glutathione-sea water solution during the irradiation period affords no radioprotection of the stolon growth inhibitory system. Perhaps the rate of movement of reduced glutathione into the animals is too slow; perhaps the tissue machinery which produces the radioprotective material is damaged by the irradiation itself, or the machinery is too slow to provide sufficient radioprotective substance in so short a time.

Incubation of colonies for 24 hours prior to irradiation does not protect the growing stolon from direct irradiation damage (series 6 and 8). The time course and events (cessation of stolon growth, squaring of the stolon tip, appearance of dents in the coenosarc, the dissolution of the coenosarc) involved in the death and destruction of the irradiated growing stolon are the same for these two series as for series 2 and 4 which were not incubated prior to irradiation. It is tempting to hypothesize that there is a specific stolon growth inhibitory substance produced by the starter, and that the production of this inhibitor substance can be decreased or eliminated by x-irradiation of the starter. The reversal of the increased rate of stolon growth by x-irradiation followed by glutathione incubation does nothing to convince us that we are dealing with a stolon growth inhibitory system rather than a stolon growth inhibitory substance. We do not, however, have any direct biochemical evidence that such an inhibitory substance exists.

We conclude that in normally growing colonies of *Campanularia flexuosa,* stolon growth consists of two functions: (1) a general growth system and (2) an inhibitory system superimposed on the first. Both functions are affected by irradiation but only the second function is protected by glutathione.

SUMMARY

1. Five- and six-day-old colonies of *Campanularia flexuosa* were subjected to x-irradiation with and without variable incubation in reduced glutathione-sea water solutions.

2. X-irradiation doses of 81,000 roentgens alone stop hydranth production and stolon growth, the latter after three days.

3. Irradiation of starters resulted in an increased rate of new stolon growth over controls.

4. Under certain conditions of incubation in glutathione-sea water prior to irradiation, there was no increase in new stolon growth when starters were irradiated.

5. An hypothesis of stolon growth control is proposed.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY 1969

ABSTRACTS OF SEMINAR PAPERS

JULY 15, 1969

Temperature-pressure effects on RNA synthesis in synchronized Tetrahymena. SHUHEI YUYAMA AND ARTHUR M. ZIMMERMAN.

In *Tetrahymena pyriformis* GL there is a change in the pattern of RNA synthesis during the period that follows the end of heat treatment (EH) and precedes synchronized cell division, although total incorporation of uridine is approximately linear during this period. Cells were pulsed with H^3-uridine for either 7 or 20 minutes starting at zero and 40 minutes after EH. RNA was extracted from these cells. The RNA was fractionated by methylated albumin Kieselguhr chromatography or by sucrose gradient centrifugation and it was found that all major classes of RNA were labeled. Radioactivity was predominantly localized in ribosomal precursor RNA and in heterogeneous high molecular weight RNA. These two RNA fractions were clearly separated immediately after EH but less clearly defined at later times. When RNA extracted from total ribosomal material was fractionated (following a 10 minute pulse), it was found that greater amounts of radioactivity were associated with ribosomal material at later times (40–50 minutes EH) than immediately after EH. Sucrose gradient analysis showed that this radioactive RNA is heterogeneous. The data suggest an increased transference of messenger RNA from nucleus to cytoplasm as the cells progress toward division.

Both high temperature and high hydrostatic pressure treatments that delay cell division inhibit total incorporation of uridine. While high temperature differentially suppresses synthesis of heterogeneous high molecular weight RNA, hydrostatic pressure suppresses synthesis of all major classes of RNA in equal proportions.

The above observations are consistent with the view that increase in ribosomal-bound RNA is linked to the synthesis of "division proteins" in the cytoplasm and that inhibition of RNA synthesis results in division delay.

This work was supported by a grant from the National Research Council of Canada.

Effects of various adenine derivatives on oocyte maturation and spawning in the starfish. HARUO KANATANI, TADASHI KUROKAWA AND KOJI NAKANISHI.

Recent investigations of the mechanism of starfish spawning have shown that an active polypeptide derived from the nervous system acts on the ovary and induces the production of a second active substance which is responsible for oocyte maturation and shedding of the gametes. The second substance has recently been isolated and identified as 1-methyladenine in *Asterias amurensis*.

Synthetic 1-methyladenine was found to be very effective in inducing oocyte maturation and spawning *in vitro* in *Asterina pectinifera, Asterias amurensis, Marthasterias glacialis, Astropecten aurantiacus* and *Ceramaster placenta*.

The effects of various adenine derivatives were also studied *in vitro* with *Asterias forbesi.* Isolated ovarian fragments were placed in Petri dishes containing 5 ml of test solutions at various concentrations for 1 hour and the degree of spawning was checked. For observing oocyte maturation, ligated ovarian fragments were exposed to the test solutions. After about 1 hour these fragments were torn with forceps and the degree of germinal vesicle breakdown was observed. 1-Methyladenine (3×10^{-7} M), 1-ethyladenine (10^{-6} M) and 1-methyladenosine

(10^{-5} *M*) were found to be very effective in inducing oocyte maturation and spawning, whereas the following compounds had no effect at concentrations from 10^{-6} *M* to 5 × 10^{-4} *M* or 10^{-3} *M*: 9-methyladenine, N_6-benzyladenine, adenine, adenosine, keinetin, 5′-AMP, cyclic AMP, 6-methylpurine, 1-methyl guanine, guanine, guanosine, 5′-GMP, inosine, 5′-IMP and hypoxanthine. From these results it is thought that a short alkyl radical such as the methyl or ethyl radical at the site of N1, and an imino radical at the site of C6 of the purine nucleus are important in inducing oocyte maturation and spawning in the starfish.

Injection of 1-methyladenine (30 μg/animal), 1-ethyladenine (33 μg/animal) or 1-methyladenosine (280 μg/animal) into the coelomic cavity caused the discharge of the gametes in male and female starfish.

Supported by the Fellowship of the Population Council, Rockefeller University.

Mercenaria *clam liver extracts: influence of the HeLa S3 cell cycle.* SISTER ARLINE C. SCHMEER, O.P.

An *in vitro* autoradiographic study was initiated to determine if *Mercenaria* clam liver extracts (called mercenene) exerted any influence on the cytokinetics of a HeLa S3 asynchronous cell population and to learn if these extracts were catabolized *in vitro*. Monolayer cultures were incubated for 20 min with ^{3}H-TdR (TZ). Five-tenths mg of mercenene/ml of media was added to the experimentals. Controls received no extract. Samples taken from TZ to 30 hr were prepared for autoradiography and the following parameters were determined: 1) the DNA synthetic index (DSI), 2) the per cent labeled mitoses (LM), and 3) the mitotic index (MI). Quastler and Sherman's techniques were employed in calculating the cell cycle from the curve describing the rate of appearance and disappearance of labeled mitoses. In the *control* cultures, LM equalled 15% by 1 hr. A plateau of 95% LM was followed by a sharp decline to 10%. A second ascending curve plateaued with 68% LM and fell to 17% at 30 hr. The mean phases, in hr, for the control cell cycle were: 1) $S = 7.5$, 2) T_{g2} + prophase = 1, 3) $M = 1$, 4) $T_{g1} = 4$, and 5) $T_g = 18$. The *experimental* curve varied significantly. No LM were scored until the 4th hr when the LM were considerably less than the controls. Maximum labeling was recorded at 65%, falling to 10% at 20 hr. A second plateau was maintained at 45% labeling. Due to the irregularity in the experimental cell cycle curve, the various phases were not estimated. The data suggest that the G_2 cells progress into mitosis at a *diminished* rate with a lag in the entire curve. The cells in S progress into G_2 at a normal rate. The DSI for the control and experimental cultures was 47.52 ± 4.21 and 47.88 ± 3.89, respectively. This suggests no direct effect on DNA synthesis. The MI for the controls and experimentals was 3.62 ± 0.22 and 4.88 ± 0.50, indicating no inhibition of cell division. Data indicates the catabolism of mercenene *in vitro*. Since inhibition in G_2 has been recorded, it is now possible to determine the mechanism involved in the inhibition of the new transcriptions observed in the HeLa cell population exposed to clam liver extracts.

This research was supported by USPHS Fellowship GM-34-903; the Arthur J. Schmitt Foundation, Chicago, Illinois; The Tad Beck Fund, Lincoln, Nebraska; and the Ohio Eagles' Stephen M. Matuschak Memorial Fund.

The "9 + 1" pattern of microtubules in spermatozoa of certain Turbellaria. C. HENLEY, D. P. COSTELLO, M. B. THOMAS AND W. D. NEWTON.

We are systematically investigating the patterns of microtubules in spermatozoa of turbellarians, including the rhabdocoel *Mesostoma georgianum* and the triclad *Dugesia tigrina*. The living spermatozoa of both forms are about 100 μ long and 0.5 μ wide, with two motile free flagella, each about 200 μ long. In electron micrographs of sections, the body of the sperm has a peripheral row of singlet cytoplasmic microtubules, just beneath the plasma membrane. In *Mesostoma* the nucleus is uniformly lamellar, but in *Dugesia* (Silveira and Porter, 1964) it has both a lamellar and a more dense portion. There are several small mitochondria in the spermatozoon of *Mesostoma*, as contrasted with the single long mitochondrion of *Dugesia* spermatozoa. Otherwise the male gametes of the two forms are very similar. The flagella have 9 peripheral doublet microtubules and a central core, *ca.* 650 Å in diameter, of complex structure.

In material negatively stained with phosphotungstic acid, the cytoplasmic singlets are *ca.* 200 Å in diameter, the doublets of the flagella *ca.* 400 Å. The central core of flagellum is composed of an innermost dense structure, around which are wound, in a double helix of 45° pitch, two hollow structures, each with a pebbly substructure in its walls. The following are rough measurements of the core components: diameter of helical hollow structures, 180 Å; center-to-center distance of pebbly structures in their walls, 80 Å, and of one gyre, 650 Å; diameter of entire core (after drying down), 720 Å. The 9 doublets, when free, tend to fall on the grids in coils of rather uniform diameter (2–4 μ). The central core often breaks away from the doublets and runs for very long distances (as much as 73 μ) in a relatively straight course. This suggests that the core has considerably more rigidity than the doublets, and may be associated with the motility of the very long flagella.

Aided by a grant from the National Institutes of Health, GM 15311.

July 22, 1969

An exact study of polygenic variation in natural populations. Roger Milkman.

The use of an allelism test, modified for application to sets of polygenes, fixed and unfixed, has demonstrated the persistence throughout the breeding season in natural populations of *Drosophila melanogaster* of certain specific alleles known for their contribution to wing vein defects.

Of over 400 lines, each derived from a single inseminated female in weekly sampling of two Amherst, Mass., populations from June to November, 1966, twelve lines have so far become true breeding, polygenic crossveinless strains in response to selection. These strains have been compared genetically by selecting the progeny of a cross between any two strains in the direction of the normal phenotype. The absence of response indicates the absence of relevant genetic variability, and therefore the identity of the two strains' sets of *cve* polygenes.

Only one pair of strains, stemming from June and November, respectively, produced progeny, when mass mated, that did not respond to back selection. These two strains had identical *fixed* sets of *cve* polygenes. But in addition, the various *cve* strains had unfixed *cve* alleles in high frequency as well. Pair matings and *upward* selection of F_1 flies produced numerous F_2's which did not respond to downward selection. Various confirmatory tests on these nonresponding lines have been completed. Thus, numerous pairs of *cve* strains share enough identical unfixed *cve* alleles to produce the maximal phenotype—and these alleles must therefore persist in the populations at substantial frequencies throughout the season.

The genic polymorphism known by precise studies to characterize the determination of numerous enzymes and antigens is now seen to apply as well to polygenes known for their collaborative morphological effects.

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Dark adaptation: its influence on GABA in frog retina, in vivo. L. T. Graham, Jr., C. F. Baxter and R. N. Lolley.

Gamma-aminobutyric acid (GABA) and glutamate were measured enzymatically in retina from light- and dark-adapted frogs (*Rana pipiens*). The frozen eyes were sectioned and lyophilized. In some experiments the dry retinal section was subdivided by microdissection into (1) a photoreceptor cell layer, (2) a bipolar cell layer also containing horizontal and amacrine cells, and (3) a ganglion cell layer. The adaptational state of the frog retina has very little influence on the content or even distribution of retinal glutamate in the cellular layers. However, dark-adapted retina contains about 50% of the GABA of light-adapted retina and the distribution of GABA in the cellular layers is affected by the state of adaptation. In the dark, GABA appears to be concentrated in the bipolar layer, while in the light, both bipolar and ganglion cell layers have equally high, increased levels of GABA. The changes are across the inner synaptic layer and thus favor an association of GABA with amacrine cells rather than with horizontal cells. Since GABA levels in the retina are altered in response to a physiological visual stimulus *in vivo*, it was speculated that the function of GABA in the retina may be related to inhibitory neural mechanisms involving amacrine cells.

Supported in part by NIH Grant NB-03743.

JULY 29, 1969

Variations in lens insoluble proteins. S. ZIGMAN.

Differences in the composition of lens insoluble proteins of several species were sought. Lenses of young and old rats, dogfish, and cows and normal and cataractous humans were separated into cortex and nucleus each of which was homogenized in water. Insoluble residues of these homogenates were washed extensively with water, and resuspended in $7\,M$ urea. The suspension was centrifuged ($100{,}000 \times g$) and the insoluble phase (UI) was sulfonated as described. The soluble phase (US) was also sulfonated after reprecipitation by dialysis. Sulfonated US and UI fractions were dissolved in water for further use.

In rat lens cortex, little change in US and UI occurred with aging. Nuclear US dropped from 85% to 25% of total insoluble protein (TIP) between 5 and 52 weeks of age while UI increased from 10% to 65%. In dogfish lens cortex, US increased from 9 to 21% of TIP, while UI fell from 26% to 4% in lenses of young and old animals respectively. The greatest change noted was the drop of US from 20 to 6% concomitant with an increase of UI from 46 to 70% in the nucleus. In the bovine lens, cortical US fell from 55% in the calf to 36% of TIP in the cow. UI also fell from 5 to 1%. Nuclear US increased from 28% in the calf to 60% in the cow, while UI increased from 1 to 3%. The distribution of US and UI in nucleus and cortex of normal human lenses (50–60 years old) is nearly identical to that in the cow, but UI was found at 70% and US at only 30% in whole cataractous lenses of the same age.

Aging and cataract formation of all lenses seems to be accompanied by a drop of γ-crystallin and a rise of α-crystallin in UI of the nucelus, and a near total loss of γ-crystallin from cortical US (by ultraviolet light extinction and acrylamide gel electrophoresis analyses). Approach to the cataractous state seems to be accompanied by a shift of US to UI predominance in the lens nucleus and by an increase in α-crystallin in cortical US and nuclear UI. The resemblances of normal human lens insoluble proteins to those of the cow, and cataractous human insoluble proteins to those of the rat and dogfish suggest that some factor at a very low level in normal human and cow lens is present in rat, dogfish and cataractous human lenses at a higher level. A factor in the blood of cataractous humans which renders gamma crystallin insoluble has been described (Testa).

Supported by research grants from the Rochester Eye Bank and the U.S.P.H.S. (#NBO 8360).

Selective antibody transport in the proximal small intestine of the neonatal rat.

RICHARD RODEWALD.

The neonatal rat selectively transports antibodies functionally unaltered from the lumen of the small intestine into the circulation. Experiments were performed to answer two questions: where along the intestine does transport take place, and what is the mechanism of selection and transport across the epithelial cells in that region. To answer the first question, segments of the intestine of 10-day-old rats were ligated *in situ* and injected with purified rat anti-ferritin γG immunoglobulin. After one hour a blood sample was taken by cardiac puncture and assayed for anti-ferritin using a passive hemagglutination test. The results indicated that the proximal one-third of the intestine is responsible for the bulk of antibody transport.

The route of transport across the epithelial cells was studied using ferritin chemically conjugated to rat γG immunoglobulin as a tracer for the electron microscope. The conjugate was selectively bound to the luminal cell surface at the base of microvilli. Transport across the cell occurred by formation of pinocytotic vesicles containing the conjugate followed by transferral of the conjugate from these vesicles to the lateral extracellular spaces by way of smaller coated vesicles. In control experiments where cells were exposed to an equal concentration of unconjugated ferritin, no tracer was found either bound to the cell surface or within any of the cytoplasmic vesicles.

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Preliminary findings on the effect of D_2O on the period of circadian activity rhythms.

John D. Palmer and H. Burgess Dowse.

Two of the most intriguing properties of the *period* of circadian rhythms are its virtual temperature independence and insensitivity to perturbation by chemical agents. In elucidating the latter characteristic a plethora of common and exotic inhibitors, tranquillizers, and anesthetics have been found to have no effect. Only in the last few years have a small number of substances been found which produce a change—one of these is deuterium oxide.

Work by others with this compound has demonstrated that the periods of the *Euglena* phototactic rhythm and the activity rhythm of the deer mouse are lengthened by its incorporation. We have extended these observations to two more species and have elucidated a further effect of D_2O on circadian rhythms.

D_2O was added to the drinking water of a sample population of African waxbills (*Estrilda troglodytes*) and their activity rhythms followed for periods of up to 65 days. Final concentrations of deuterium varied between 10 and 35% in 5% intervals. At all concentrations the periods of the activity rhythms of the deuterated birds were increased as a direct function of the concentration of deuterium in the drinking water. At the highest concentration the average increase was 7%.

A similar set of observations were made on the common laboratory mouse (*Mus musculus*) with the same results: increasing deuteration produced longer periods—6% being the average maximum increase at 30% D_2O. Quite unexpectedly it was found that under the influence of 20% deuterium in the drinking water, the mouse activity rhythm was no longer entrained by alternating light-dark cycles (12 hr light: 12 hr dark). On return to protiated water the locomotor rhythm was gradually entrained again by the light-dark cycles.

The actual mode of action of deuterium on the biological clock controlling these rhythms is not yet understood.

August 5, 1969

Photoregulation of bioelectricity by photochromic acetylcholine receptor inhibitors.

Walter J. Deal.

The photochromic compounds N-p-phenylazophenyl-N-phenylcarbamycholine chloride and p-phenylazophenyltrimethyl-ammonium chloride inhibit the carbamylcholine-produced depolarization of the excitable membrane of the monocellular electroplax preparation of *Electrophorus*. The *trans* isomer of each predominates in the light of a photoflood (420 mμ) lamp; they are stronger inhibitors than the *cis* isomers, which predominate under ultraviolet (320 mμ) irradiation. The potential difference across the excitable membrane may be photoregulated by exposing an electroplax in the presence of a solution of carbamylcholine and either of the two compounds to light of appropriate wave-lengths, since light shifts the *cis-trans* equilibrium. The system may be considered as a model illustrating how one may link a *cis-trans* isomerization, the first step in the initiation of a visual impulse, with substantial changes (20–30 millivolts) in the potential difference across an excitable membrane.

Studies on the conformation of small molecules acting on acetylcholine receptors.

Henry G. Mautner and Eli Shefter.

It has been proposed that acetylcholine (AcCh) is the trigger molecule attachment of which to membrane receptors induces a conformational change altering membrane permeability. The difficulties in interpreting such conformational changes are emphasized by the fact that even the conformations of the trigger molecules were ill defined until recently.

In 1966 it was shown by x-ray diffraction and NMR studies that the -N-C-C-O-grouping in AcCh is in the *gauche* (*sc*) conformation both in the crystal and in D_2O solution. Since then, it was demonstrated the replacement of the acyloxy oxygen of AcCh with sulfur and selenium induced a major conformational change, acetylthiolcholine and acetylselenolcholine being in the extended *trans* (*ap*) conformation. Since the latter compounds are essentially isosteric but have widely different depolarizing activities, electronic factors must play as important a role as conformational factors in the actions of such compounds. On the other hand, replacement of the carbonyl oxygen of AcCh with sulfur does not alter the *sc* conformation of the molecule.

The abilities of AcCh and acetylthionocholine to induce depolarization in the electroplax preparation are very similar. Hydrolysis of acetylthionocholine by acetylcholinesterase yields thionoacyl-enzyme, deacylation of which is rate-limiting.

It seems that interaction between the quaternary nitrogen of AcCh with the acyloxy but not with the carbonyl oxygen is primarily responsible for the conformation of this molecule. However, the high depolarizing activity of molecules that maintain the *ap* conformation in the crystal or in solution, such as acetylthiolcholine or cholinethiol, suggests that electronic factors and the ability to undergo lipophilic interactions are of primary importance for the biological actions of such compounds. More work on the conformational flexibility of AcCh analogs will be needed, however, before definite conclusions can be drawn.

This work was supported by grants from the National Science Foundation (GB-6835), the National Institute of Neurological Diseases (NB-07835) (H. G. M.) and the National Institute of Cancer (CA-10104) (E. S.).

Some properties of junctional cilia in molluscan gills. MICHAEL J. GREENBERG.

The gill filaments of filibranch bivalves are held in register by interfilamentary ciliary junctions—patches of interdigitating cilia. These junctional cilia (J-cilia), in *Aequipecten irradians,* are borne on muscular tongue-like structures, each overlapping a similar tongue on the adjacent filament. J-cilia in the center of a junctional region are quiescent or slightly vibratile; peripherally, or on decoupled tongues, they writhe randomly.

Aequipecten ciliary junctions were studied by transmission and scanning electron microscopy. J-cilia and motile cilia have similar diameters (0.2 μ), but J-cilia are short (6.5 μ)—the width of the junction. Most J-cilia are paired throughout their lengths, although single cilia and triads also occur. A gap of about 80 Å separates the apposed outer membranes of paired cilia; no modification of the unit structure was seen. The two cilia of a pair may arise either from opposite sides of the junction, or from adjacent basal granules on the same side. Furthermore, most J-cilia on uncoupled tongues are paired. Possibly, pairing is a dynamic process involving continuous coupling and uncoupling; cilia of decoupled tongues would then pair with each other while, if two such tongues were brought together, heterofilament pairs would begin to form. Finally, any junction should contain single cilia, as well as heterofilament and homofilament pairs.

Isolated filaments, prepared by teasing or shaking the gill apart, move randomly, propelled by the feeding cilia. Coupling occurs only when J-ciliated tongues on the same, or separate, filaments touch; feeding cilia attach neither to J-cilia nor to each other. Ciliary interaction is not species specific; junctions were effected between isolated filaments of *A. irradians* and *A. muscosus.* The interfamily linkage: *A. irradians* × *Modiolus demissus* was also made. The unique properties of J-cilia probably reflect the chemical composition of their surface membranes. The lack of coupling specificity suggests a similar composition in other compound cilia.

AUGUST 12, 1969

Possible significance of "DFP-ase" in squid nerve. F. C. G. HOSKIN.

The potent inhibitor of cholinesterase, diisopropylphosphorofluoridate (DFP) is rapidly detoxified by a hydrolytic enzyme found in high concentration in the giant axon of the squid, *Loligo pealei.* Preparatory to purification of this enzyme, other tissue has been examined as a richer or more plentiful source. Both of these criteria have been met in the squid head ganglion. Whereas the giant axon hydrolyzes 50-75 μmoles DFP per g homogenized fresh tissue per hour, the head ganglian hydrolyzes 450–500 μmoles DFP per g per hour.

Although the enzyme has been tentatively identified with Augustinsson's phosphorylphosphatase, trivially called "DFPase," one property of that enzyme appears to be missing, namely a significant stimulation by Mn^{++}. In view of Aldridge's evidence that a mammalian serum esterase (A-esterase) hydrolyzes a similar organophosphate, diethyl *p*-nitrophenyl phosphate (paraoxon) as well as *p*-nitrophenyl acetate, the squid enzyme was tested for its possible identity with A-esterase. A high rate of hydrolysis of *p*-nitrophenyl acetate by squid ganglion was indeed found, but it was markedly inhibited by DFP, indicating that the DFP-hydrolyzing enzyme of squid may still be tentatively termed DFPase, and that, in addition, squid nerve is rich in an enzyme resembling Aldridge's B-esterase. This latter enzyme may be responsible for hydrolysis of diethylaminoethyl *p*-aminobenzoate (procaine), thus possibly accounting for some unusual findings with respect to uptake of this local anesthetic. The presence of these two enzymes, DFPase and B-esterase, may have important consequences with respect to the effects of organophosphorus insecticides and nerve gases, and with respect to local anesthetic action, on nerve function.

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The Goldman equation: its constraints and its applicability in the face of electrogenic and non-electrogenic pumps. TOBIAS L. SCHWARTZ.

The study of membrane phenomena requires an easily usable relationship between the transmembrane potential and the ionic constituents of the bounding fluids which can be justified on theoretical grounds. The Goldman equation is, perhaps, the most used of the available expressions because of its simplicity. However, it has often been applied to experimental situations in contradiction with the constraints required in its derivation. In the present work the Goldman equation is rederived in an attempt to minimize the necessary constraints. The membrane is treated as a Nernst-Planck diffusion regime. The discussion is restricted to univalent species, and the free diffusion zero current, steady state case. It is found that the constraint of constant mobility as one crosses the membrane cannot be discarded without generating permeabilities that may be functions of membrane potential. The equation does not therefore seem to apply to epithelial membranes. It is found, in agreement with other workers, that a class of either symmetric or asymmetric electric fields can be defined that allow the expansion to be valid. Furthermore, for ions of one sign only no specification of the electric field is required at all. The constant field constraint is thus confirmed to be too restrictive. In the presence of active fluxes two different types of steady states must be distinguished corresponding to whether the active and passive paths are spatially separate or spatially inseparable in the membrane interior. In the former situation both the active and passive fluxes are individually constrained to a steady state. In the latter case only the total flux is so constrained. When the paths are separate it is shown that a nonelectrogenic pump cannot affect the membrane potential. Surprisingly, however, when the paths are inseparable a nonelectrogenic pump will in general directly alter the membrane potential.

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Application of continuous particle electrophoresis for the separation of lipoproteins having melanin-dispersing activity from eyestalks of fiddler crabs (Uca pugilator). CLELMER K. BARTELL AND MILTON FINGERMAN.

Three-hundred eyestalks were extracted in ethanol and centrifuged. The liquid portion was decanted, and after the alcohol evaporated, the residue was suspended in $2.5 \times 10^{-4}\,M$ barbital buffer, pH 7.6. A lipoprotein fraction, having most of the melanin-dispersing activity, was obtained by ultracentrifugation of the suspended residue. The resulting pellet was resuspended in 0.4 ml of buffer and injected at 40 μl/minute into the 48 mm-wide buffer curtain of a Beckman Continuous Particle Electrophoresis System. Electrophoresis was effected at 48 volts/cm, 8 ma and a curtain buffer flow rate of 20 ml/minute. The front plate was cooled by circulating water at 4° C. After the run each of the 48 samples was concentrated by centrifugation and the pellets recovered. Each pellet was suspended in 1 ml of distilled water, and an aliquot adjusted to physiological tonicity and assayed in eyestalkless *U. pugilator* for melanin-dispersing activity.

Two prominent peaks of biological activity, at 13 and 17 mm from the origin towards the positive electrode, corresponded to the absorption peaks determined at 460 mμ (the absorption peak for carotenoids dissolved in the lipid phase of the lipoprotein micelles). Chemical analyses of the two active fractions were as follows: Total lipids (N-butanol-soluble fraction, by weight), at 13 mm, 80%; at 17 mm, 87%. Total protein (Lowery), at 13 mm, 20%; at 17 mm, 13%. No reducing sugars were detected (DNSA test). Electron microscopy of preparations from the material at 13 mm, fixed with osmium fumes and evaporated onto Formvar-coated grids, indicated a granular material, the repeating unit being about 130 to 170 Å. Granular units of the same size were also present as spheroid aggregates of from 6000 to 11,000 Å. The active lipoprotein material is in a density range between 1.01 and 1.03 g/ml as determined by ultracentrifugation. Lipoproteins of this density class from human sera are reported to be of a size similar to the small units seen here.

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GENERAL SCIENTIFIC MEETINGS

August 18–21, 1969

Abstracts in this section are arranged alphabetically by author. Author and subject references will be found also in the regular volume index, appearing in the December issue.

An unusual filopodial amoeba from the Eel Pond, Woods Hole. Robert D. Allen and R.W. Drum.

An unidentified spindle-shaped filopodial amoeba is reported here because of its unusual manner of movement. Extremely thin and too transparent to be seen in the bright-field microscope, it may have escaped attention in the past.

Found in the intertidal zone near a freshwater inlet to the Eel Pond on School Street, Woods Hole, the organism measured up to 40 μ in length and characteristically moved by a single leading filopodium 20 μ long. The body was *ca.* 10 μ long and 1.0–1.5 μ in diameter and contained one large ovoid inclusion, assumed to be the nucleus (1.2 × 0.8 μ) and fewer than a half dozen other resolvable inclusions.

Locomotion for several minutes at a time was by activity of the leading filopodium, which was no thicker than 0.5 μ. Both the filopodium and the barely visible particles streaming within it, showed a Brownian component. The leading filopodium was characteristically straight, but showed an ability to bend sharply at acute angles without affecting particle motions. Particles barely visible with an oil immersion 40 × achromat (0.85 N.A.) Nomarski differential interference contrast system moved in perfect order out to the tip, made a 180° "U turn" and returned to the body. Branch pseudopods formed, and their bases travelled back and forth along the main filopod and body. Periodically the organism lost its polarity and became more polypodial, the pseudopods becoming thicker, flaccid and hyaline.

The organism may be either an amoeba or a juvenile state of a foraminiferan. In either case, alternation between characteristic foraminiferan and amoeboid modes of pseudopod formation is of considerable theoretical interest.

Effect of fertilization on the uptake and efflux of calcium-45 in the eggs of Arbacia punctulata. Roobik Azarnia and Edward L. Chambers.

To two 0.2% suspensions of jelly-free unfertilized eggs in normal sea water (20° C) was added 0.1 mC ^{45}Ca/100 ml, and one of the suspensions was inseminated. Samples were removed at intervals, washed 3 times with cold sea water, and radioactivity of the eggs counted in a Packard spectrometer. The uptake of ^{45}Ca by unfertilized eggs is linear and equivalent to an influx of 200 pmoles/ml eggs/min. Fertilization results in an immediate increase in uptake of ^{45}Ca initially equivalent to an influx of 10,000 pmoles Ca/ml eggs/min. The early rapid phase has a duration of approximately 80 min and is followed by a continuing phase of slow ^{45}Ca uptake.

Unfertilized eggs were prelabeled with ^{45}Ca by suspending them for 24 hours in sea water containing 0.1 mC ^{45}Ca/100 ml and 0.1% streptomycin. The eggs were washed 3 times, suspended at a concentration of 0.02% in radioactive-free sea water, and divided into two suspensions, one of which was inseminated. Efflux from the unfertilized eggs occurred at an exceedingly slow rate equivalent to an exchange of 0.4% of the total cell calcium per hour. After fertilization, a phase of rapid efflux was observed followed by a far slower phase. The rapidly exchanging component constituting 30% of the total intracellular calcium had a half-time of 15 min, while the slowly exchanging component comprising the remaining 70% had a half-time of 220 min.

The efflux of total calcium and ^{45}Ca were measured simultaneously in Ca-free sea water. The efflux of ^{45}Ca was the same as in normal sea water except for a slight slowing during the slow phase. The decrease in calcium content of the eggs (initially 3 μm/ml eggs) paralleled the efflux of ^{45}Ca, indicating that the efflux of the isotope reliably measures the exchange of of total calcium. In conclusion, the data show that fertilization strikingly increases the rate of exchange of the intracellular calcium.

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The fine structure of the photoreceptor of the ascidian, Amaroucium constellatum.

STEPHEN N. BARNES.

The eye of the free-swimming tadpole larvae of the tunicate, *Amaroucium constellatum,* has shown with the light microscope to consist of three parts: the lens cells, the pigment cup, and the retinal cells. For electron microscopy the tadpoles were fixed in 6 per cent glutaraldehyde in 0.2 *M* s-collidine buffer, pH 7.4 (3 hours), rinsed in buffer, and post-fixed in 1 per cent osmium tetroxide in 0.1 *M* Sorensen's phosphate buffer, pH 7.4 (1 hour). The osmolality of all three solutions was adjusted to 1200 milliosmoles with sucrose. This report describes the fine structure of the three parts of the eye.

There are at least three melanin-containing pigment cells which form a pigment cup. This cup functions as a light shield, thus allowing the tadpole to respond directionally to light. No unusual morphological features of these cells have been noticed.

The three lens cells lie against the epidermis and extend down into the sensory vesicle; they are joined to one another by desmosomes. Their largest volume is occupied by a clear material, probably the mucopolysaccharide tunicin; this region is bounded by mitochondria. The remainder of each cell is filled with a dense, homogeneous cytoplasm and a nucleus. The function of the lens cells is unclear.

The seven to ten photoreceptive cells (maximum diameter 20 microns) send processes through the pigment cup. The ends of these processes differentiate into the photoreceptive membranes which lie between the pigment cup and the lens cells. These photoreceptive membranes arise as lamellae from the membrane of a cilium, thus this is a ciliary photoreceptor. The base of each photoreceptive cell contains a nucleus, mitochondria, groups of neurosecretory granules, and encloses a nerve bundle which passes to the ganglion. No synapses have been seen at the base of the photoreceptive cells.

Preliminary observations on the aggregation of the amebocytes of the horseshoe crab Limulus polyphemus.

FRANK A. BELAMARICH, DAVID SHEPRO AND DIANNE KENNEY.

When hemolymph (blood) is removed from *Limulus* the amebocytes rapidly aggregate. The rate and degree of aggregation can be measured photometrically by recording the changes in turbidity as per cent transmittance.

Disodium ethylenediaminetetraacetate (EDTA) and trisodium citrate (80 m*M*) partially inhibited aggregation, and N-ethylmaleimide (NEM) and parahydroxymercuribenzoate at similar concentrations produced almost complete inhibition. Low temperatures (0° C) also produced partial inhibition. The EDTA or citrate inhibition was not reversed by the addition of high concentrations of calcium or magnesium, nor was it reversed by the addition of plasma made by centrifuging 0° C blood for 1 min at 8000 × g. Inhibition was reversed by the addition of 'serum,' the supernatant of a centrifuged cellular clot, and the supernatant of a homogenate of plasma-free cells suspended in buffered 0.51 *M* NaCl. The active substances in the serum and cell homogenate supernatant were nondialyzable and heat-labile. The cell homogenate supernatant retained some portion of its activity when heated.

When serum or cell homogenate supernatant were reacted with NEM or EDTA and dialyzed, the serum and supernatant retained their ability to reverse the EDTA inhibition. Serum and supernatant did not reverse NEM inhibition even when the cells were resuspended in buffered saline. Trypsin reversed EDTA inhibition.

The data indicate the presence of an aggregating substance in the amebocytes of *Limulus* blood.

This research was supported by grants from the National Heart Institute (HE10002) and the American Heart Association (67–690).

A fixation technique for electron microscopy which provides uniformly good preservation of the tissues of a variety of marine invertebrates. Allen L. Bell, Stephan N. Barnes and Karen L. Anderson.

While studying the fine structure of the eyes of some molluscs and ascidian tadpoles, we developed a fixative which yielded excellent preservation of these tissues for electron microscopy. The method was discovered empirically by trying a large number of different combinations of fixing agents and buffers, and by varying the osmolality of each fixing fluid.

The fixative that gave consistently good results was 6.0 per cent glutaraldehyde in 0.2 Molar s-collidine buffer at pH 7.4 to which sucrose was added to bring the osmolality up to 1200 milliosmoles. The final sucrose concentration necessary to reach 1200 milliosmoles was 8.6 per cent. A trace of calcium chloride was dissolved in the final mixture.

Fixation was initiated at room temperature, continued in a refrigerator overnight, rinsed in buffer the next morning and postfixed. Postfixation was carried out in 1.0 per cent osmium tetroxide in phosphate buffer at pH 7.4. The osmolality of the buffer rinse and the postfixing solution was also brought to 1200 milliosmoles with sucrose.

To date we have successfully tried this fixative on the following marine invertebrate tissues. *Aequipecten irradians:* eye and ovary; *Lima scabra:* eye; *Thyone briareus:* intestine; *Amaroucium constellatum:* whole animal; *Uca pugnax:* rectum; *Metridium senile:* siphonoglyph; and, *Magelona sp.:* whole animal.

Serial reconstruction of fine-structural interrelationships in the germarium of Habrobracon juglandis. Joseph D. Cassidy, O.P. and Robert C. King.

In the developing braconid, cellular interconnections have provided a key to cell lineage. This survey examined the ultrastructural changes resulting in the formation of the egg chamber in Whiting stock 33. High-resolution electron microscopy of 600–700 Å serial sections obtained with the LKB Ultratome-III followed fixation in cacodylate-buffered glutaraldehyde and OsO_4. Montages consisting of Kodak positive sheet films were constructed from composite series of electron micrographs. Three-dimensional reconstruction of the germarial region localized the associations of the developing ova, their accessory nuclei, prefollicular mesoderm, smooth muscle and lumen cells. The presence of multi-nucleolate stem line oogonia, and the identification of pro-oocytes containing synaptonemal complexes were documented.

Throughout the animal kingdom, during oogenesis, germ cells are commonly found to be bridged by cytoplasmic canals at an early stage in their development. In serial transverse section, the rim surrounding each ring canal of *Habrobracon* cell clusters is made up of 8 leaves arranged in four pairs. Each leaf consists of a monolayer of short parallel microtubules. The leaves of each pair overlap with the leaves of adjacent pairs. The sliding of four leaves past one another allows the ring to dilate as the cystocyte grows. Four short, broad leaves have a length of 1.1 μ and a width of 0.7 μ while the four long, narrow leaves have a length of 2.0 μ and a width of 0.4 μ. The mean outer diameter measures 200 Å and each is separated from its neighbor by a space 100 Å wide. Each short and long leaf contains 40 and 70 microtubules, respectively, embedded in an electron-dense matrix. The origin of this system prior to the onset of cytokinesis is consistent with the interpretation that the ring canal derives from parallel tubules in the mitotic apparatus, *i.e.*, the mid-body of the spindle, rather than arising from the cleavage furrow.

Supported by USPHS Grant 5T1GM903–7 and NSF Grant GB-7457.

A chemical signal attracting the flatworm Bdelloura candida *to its host,* Limulus polyphemus.

ROBERT L. CHEVALIER AND H. BURR STEINBACH.

It has been known for over a century that a small triclad, *Bdelloura candida,* is present in large numbers only on the legs and gills of *Limulus polyphemus.* The present study was a preliminary attempt to discover the means by which *Bdelloura* identifies its host. Whether it is a parasite or commensal has not been established, but it was found that *Bdelloura* will move toward a current of sea water coming from a vessel containing *Limulus* but not toward water bathing *Libinia, Cancer,* or *Pagurus.* This indicates the presence of some specific chemical factor released into the water which is detected by the worms. A simple glass Y-tube choice apparatus was used in an assay of the "host-factor." When enough factor is present to be detected by the worms, they will move up the arm of its source, never entering the other arm more than once in ten trials. As soon as the concentration falls below this threshold, *Bdelloura* will not move against the current.

In attempts to characterize the factor, it was found to be stable in sea water for at least five days, and remained active even after evaporation to dryness at 45° C. Bringing the solution to a boil does not destroy all activity, although boiling for 15 minutes or more eliminates activity. The factor penetrates a dialyzing membrane, indicating a relatively low molecular weight. Establishing the source of the factor proved difficult because a damaged *Limulus* was found to release some inhibitory substance repelling *Bdelloura.* Water extracts of *Limulus* blood, isolated legs and gills, and brick-red glands proved inactive or inhibitory.

It is hoped that these preliminary results will stimulate a biochemical approach to isolating and analyzing the chemical signal(s) involved.

Experimental inhibition of ciliogenesis and ciliary regeneration in Arbacia *embryos.*

FRANK M. CHILD AND MATTHEW N. APTER.

The formation of motile cilia during ciliogenesis or ciliary regeneration in *Arbacia* blastulae may be conveniently assayed by allowing a sufficient number of particles of carmine to settle upon the blastulae, and then scoring the percentage of embryos which are visibly jiggling the carmine (% CJT). Beginning about 5 hr after fertilization, the CJT rises from zero to 100% in about 45 min, in demembranated embryos at 24° C. If cilia of blastulae are amputated and allowed to regenerate, then beginning about 21 min after amputation at 24° C the CJT rises from zero to 100% in about 8 min.

Pactamycin at concentrations between 20 and 100 $\mu g \cdot ml^{-1}$ delays the scheduled time of rise of CJT during both ciliogenesis and ciliary regeneration if added sufficiently early. The amount of delay varies with the time of addition and concentration of pactamycin and with batches of embryos. Pactamycin does not paralyze motile cilia; neither does it prevent the expected onset of the rise of CJT when added a minute or two before the rise is scheduled. Hence, pactamycin does not *directly* inhibit the *assembly* of substances into forming cilia, at least within the limits of the CJT assay.

Pactamycin inhibits the rate of incorporation of ^{14}C-valine into total protein of intact blastulae, *e.g.,* 50 $\mu g \cdot ml^{-1}$ inhibited the rate 94%. If we assume that pactamycin has no significant effect other than inhibition of protein synthesis, then there must exist at least one protein the synthesis of which is necessary sometime during the several minutes preceding the scheduled onset of rise of CJT, in certain cells of the blastulae. This conclusion suggests that the synthesis of this protein(s) in these cells is a controlling event in the development of

CJT, *i.e.,* the formation of motile cilia. We do not know if this protein assembles into the structure of the cilia or rather promotes the assembly of other components.

Work supported by NIH grant GM-13993; pactamycin gift of NCI.

The degranulation and motility of amoebocytes from Limulus *blood during coagulation.* ANDREA CLARK, JACK LEVIN AND ROBERT D. ALLEN.

Motion picture records were made showing the behavior of amoebocytes in *Limulus* blood, both in living gill tissue and in blood withdrawn within one or two seconds before it was placed in a slide and filmed.

Amoebocytes in gill tissue are packed with highly refractile (phase retarding) granules and move with eruptive hyaline lopodia at speeds comparable with free-living *"limax"* amoebae.

When blood is removed and instantly observed on a slide, it clots, and the cells lose their refractile granules. This degranulation requires about two minutes. During this time the amoebocytes completely alter their morphology and manner of movement. They flatten and spread, extending filopodia ("microspikes") and pharopodia ("hyaline veils"). Very few cells exhibit amoeboid progression at velocities comparable with granulated cells in gill tissue.

Amoebocytes have been shown to contain all of the clottable protein of *Limulus* blood. The granules disappear explosively during coagulation, suggesting that they contain the clottable protein and/or other factors required for blood coagulation.

Some aspects of sperm-egg interaction in Cerebratulus lacteus. WALLIS H. CLARK, JR. AND GERTRUDE W. HINSCH.

The head of the *Cerebratulus lacteus* sperm is long and slightly sickle-shaped. It contains an elongate nucleus and apically a well defined acrosome. An aggregate of electron dense material appears between the nucleus and acrosome. An asymmetrical midpiece consists of five mitochondria which surround a narrow posterior portion of the nucleus, a proximal centriole and a prominent centriolar satellite associated with the basal portion of the flagellar tubules.

Early workers using the light microscope reported that the sperm easily and rapidly penetrated the vitelline coat and egg surface during sperm-egg interaction. No acrosomal reaction or sperm lysins were reported.

The present ultrastructural observations of polyspermic eggs fertilized prior to germinal vesicle breakdown indicates that sperm penetrate the vitelline coat without undergoing any observable change in the acrosomal vesicle. The intact apical end of the sperm produces an indentation in the oolemma. The initial stages of membrane fusion and the acrosomal reaction, which presumably takes place just prior to fusion, have not as yet been observed. Subsequent to gamete plasma membrane fusion the sperm's nucleus, with intact nuclear membranes, midpiece and portions of the flagellum enter the ooplasm of the egg.

This study was performed in the Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory (NIH grant T1-HD-26-08).

All-or-none excitation and the squid axon membrane. K. S. COLE, R. GUTTMAN AND F. BEZANILLA.

Spatially uniform membrane potentials and current densities were first produced in the squid axon with internal and external electrode arrangements and later by isolating a short length of axon between external pools of sucrose. Earlier stimulus-response characteristics between 5° C and 23° C have been supplemented and extended by measurements from 15° C to 38° C. Below 20° C or 25° C excitation appeared to be all-or-none to within limits set by spontaneous fluctuations. But with increasing temperatures it became easier to obtain intermediate responses.

Based on the empirical sodium and potassium conductances given by Hodgkin and Huxley, calculations for the spatially uniform membrane at 6.3° C showed the response to be graded but only over a stimulus range of 10^{-7} mV. This seemed entirely academic because the membrane was obviously all-or-none for any practical purpose. From further calculations the maximum slope of the stimulus-response curve decreases from 10^{9} to 500 to 6 as the temperature is raised from 6.3° C to 20° C to 40° C. These calculations are in reasonable agreement with experiments on several axons at 15° C and at 35° C. It may be concluded that the all-or-none law does not apply to the spatially uniform membrane. However previous calculations for impulse initiation and propagation along an axon were in excellent agreement with experiment and followed the all-or-none law.

These measurements and calculations provide further support for the conductances as important parameters of membrane structure and normal operation. They are continuous with continuous derivatives as functions of potential, time and temperature and it seems unlikely that abrupt, threshold, all-or-none, or transition phenomena are major factors in the processes by which ions cross the normal squid axon membrane.

Activation of pigment dispersion in melanophores of the fiddler crab (Uca pugilator) *by the near ultraviolet.* THOMAS P. COOHILL, CLELMER K. BARTELL AND MILTON FINGERMAN.

When melanophores of destalked fiddler crabs, or of isolated legs, are exposed to sunlight, pigment dispersion occurs as a direct response to light. The object of the present experiment was to determine the relative efficiences of three broad spectral ranges of light in effecting pigment dispersion in melanophores *in situ*. Destalked crabs (males, 17 mm carapace width), with melanin concentrated, were shielded so that the walking legs on one side were exposed to light while the remainder of the animal was shielded with aluminum foil. The legs shielded from light served as controls.

Light of wavelengths shorter than 3000 Å (2537 Å Germicidal lamp) elicited no response up to 1.37×10^5 μw/cm^2. The cuticle does not transmit light below 3000 Å. Light in the near ultraviolet (3000 to 4000 Å) was the most effective. A dose of about 50,000 μw/cm^2 (in 2 minutes) produced melanin dispersion to stage 3 (Hogben-Slome scale), the response lasting up to 20 minutes following the end of light exposure. About 20% of the near ultraviolet was transmitted by the cuticle. Doses in this wavelength range up to a dose of 150,000 μw/cm^2 (in 2 minutes) did not increase the response. Below 50,000 μw/cm^2 the response decreased with decreasing dosage. After 70 minutes and a total dose of 1.44×10^6 μw/cm^2 the pigment reached maximum dispersion and within 30 minutes of darkness the pigment became maximally concentrated. Larger dose rates for shorter periods (less than one minute) were not quite as effective as lower dose rates for longer periods (up to two minutes) for the same total dose. Visible light (4000 to 7000 Å) was about 200 times less efficient than near ultraviolet in eliciting equivalent responses. The cuticle transmits about 72% of the visible light. The direct response to light in the near ultraviolet may be of adaptive value in serving to shield internal organs from direct sunlight.

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In vitro incorporation of yttrium-90 by spermatozoa of marine invertebrates. ROGER K. COOMES AND LEONARD NELSON.

In this study, spermatozoa from *Metridium, Arbacia, Asterias* and *Spisula* were tested to determine their ability to incorporate radioyttrium. Sperm were incubated for a two hour period in 20 ml of buffered calcium-magnesium sea-water which contained 1 μCi per ml ^{90}Y. Serial sperm samples were removed at selected time intervals from 1 to 120 minutes, the sperm deposited on HAWP Millipore filters, rinsed and analyzed for radioactivity with a liquid scintillation counter using the method of V. K. Haberer. Unlike mammalian spermatozoa, incubated *in vitro* under similar conditions, sperm from the invertebrate species removed very little yttrium from the incubation medium. The highest level of incorporation found was 1.7% of the dose per ml removed by *Asterias* spermatozoa (5×10^6 sperm/ml). After three hours of incubation, these sperm were still motile and could induce fertilization membrane formation and cleavage with activated *Asterias* eggs.

To investigate yttrium incorporation differences between mammalian and invertebrate sperm, physical and biological factors were examined. Yttrium-90 in pH 7 and pH 8.2 buffered seawater was centrifuged on a 5 to 20% sucrose gradient for 90 minutes at $51{,}000 \times g$. It was found that larger sized colloidal particles were formed in the pH 8.2 medium. Electron microscopy of stable yttrium-89 colloids plated on coated grids supported the centrifugation results. Since W. A. Anderson has reported that *Arbacia* sperm possess a ruthenium red stainable surface coat of acid mucopolysaccharide material, an attempt was made to remove this coat. *Asterias* sperm were pre-treated for 10 minutes in 0.01% Triton X-100 before incubation with ^{90}Y. Nuclide incorporation was increased by a factor of 10. Electron micrographs of ruthenium stained *Asterias* sperm suggested that there was also a diminution of surface stainability after the Triton X-100 treatment.

This study was performed in the Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory (NIH grant T1-HD-26-08).

Straightening, bending, and spiraling in glutaraldehyde-fixed spermatozoa: a reactivation phenomenon. DONALD P. COSTELLO AND CATHERINE HENLEY.

When living "dry" spermatozoa of *Chaetopterus* are treated with fresh 1% phosphotungstic acid at pH 6.8, an initial effect is that the tails snap into multiple coils around the heads of the spermatozoa. Maceration of flagellar membrane and matrix then takes place (in the absence of the divalent ions of sea water). If samples are transferred to coated EM grids at intervals, and the fluid suddenly withdrawn, negatively stained preparations of the $9+2$ axoneme microtubules, in various stages of maceration and separation, can be demonstrated by electron microscopy. The coiling (or spiraling) of the doublet microtubules in 2 to 4 micron circles around the electron-opaque sperm head is characteristic of these preparations.

"Dry" spermatozoa of *Chaetopterus*, fixed in a cacodylate-buffered glutaraldehyde fixative (devised for acoel flatworms), do not coagulate into a mass but are fixed, in suspension, in essentially straight form. Diluting these fixed spermatozoa with buffer-sucrose wash does not change this form. Diluting the glutaraldehyde-fixed spermatozoa with the PTA solution, however, results in a reactivation phenomenon, taking place over an interval of 20 to 30 or more minutes. The tails become curved, looped, and/or helical, as if waves were being propagated along the length of the flagellum. The form of the "wave" is clearly not planar, but helical or spiral. The form changes so slowly that actual movement of flagella or spermatozoa is not perceptible by phase microscopy. Spermatozoa of *Chaetopterus* that have been in the fixative three weeks can still be reactivated, but in a less spectacular fashion. ATP or GTP, or Salyrgan, similarly applied, produced no response. However, 4% PTA, at approximate pH 2, or $HgCl_2$, produced excellent tail spiraling. Space prohibits the listing of other agents which were found to be ineffective. So far, we have been unable to reverse the coiling with specific agents, but tight helices eventually relax spontaneously with the passage of time.

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The synthesis of RNA during early development of Arbacia punctulata *embryos.* G. H. COUSINEAU, R. DAIGNEAULT, G. BELLEMARE, P. L. KRUPA AND SADAYUKI INOUÉ.

Incorporation of P^{32} into ribonucleic acids of developing *Arabacia punctulata* embryos was measured. Total RNA, extracted by the hot and cold phenol-SDS method, was placed onto 7.5% and 2.4% polyacrylamide gels in Quartz tubes, and electrophoresis was carried out in a Buchler Poly-Analyst. The RNA profiles were obtained by scanning the gels at 260 mμ in a Schoeffel microspectrodensitometer. The gels were then frozen, cut, and the radioactivity monitored in a low background surface counter. The counts obtained from these experiments did not reveal appreciable RNA synthesis until 8 hours after fertilization. At that time an important amount of radioactivity was associated with the 4S RNA, and incorporation into this species increased with development. Twelve hours after fertilization peaks of radioactivity were observed in the 36S and 32S regions, but the O.D. scan for these peaks was approximately at the base line. Similar results were also obtained at 15, 18 and 24 hours following fertilization, however, during this period synthesis of 26S, 23S and 18S RNAs also occurred. In the stages between the unfertilized eggs and the 48 hours-old embryos that were studied the O.D. profiles showed the presence of the following RNA species: 26S, 23S, 18S, approximately 12S and 9S, 5S and 4S. Studies are continuing on the characterization of the 12S and 9S species, the meaning of the 4S labeling, and the significance of the 36S and 32S precursor molecules.

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Evidence for a cooperative, reversible reaction and an irreversible reaction of TTX with the squid axon membrane. D. G. Davis, Y. Palti and W. J. Adelman, Jr.

Squid giant axons were voltage clamped in normal artificial sea water (ASW) and in ASW containing TTX in concentrations of 1 to 25 n*M*. Peak sodium, I_{Na}, initiated upon depolarization from the resting potential was measured as a function of time after the application of TTX. Measurements were continued during TTX washout, reapplication of TTX and subsequent washout. The decrease in I_{Na} after the first application of TTX was only partially recovered. The fraction of non-recovered I_{Na} (*ca.* 0.25), which was considerably larger than that attributed to axon deterioration, was found to be independent of TTX concentration. A second application of TTX did not increase the fraction of irreversible or non-recoverable I_{Na}. These results indicate there are at least two modes of TTX reaction with membrane sites. This may be due to two different active chemical species of TTX or two different loci of TTX action.

Analysis of the equilibrium behavior of the reversible fraction of I_{Na} shows that the equilibrium constant and hence the free energy of the TTX reaction with the membrane sites is a function of the fraction of TTX bound sites. This finding can be interpreted as evidence for cooperative interactions between TTX binding sites and that the interaction energy between sites is on the order of 2 to 3 kcal/mole.

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Receptive field studies of single units in the optic lobe of the squid, Loligo pealei. N. W. Daw and A. L. Pearlman.

The eye and optic lobe of the squid offer several anatomical features which are of interest in the study of the neuronal organization of the visual system. In a preliminary study, recordings were made from single units in the optic nerve and optic lobe of the squid. The animal was prepared by a method based on those of MacNichol and Love, and Wurtz. It was anaesthetized with 0.3% urethane, then held down by four pins through the head, and lead jackets over the mantle and tentacles. After cutting a small hole through the muscle for insertion of a tungsten microelectrode, anaesthesia was stopped, and the animal was maintained by running oxygenated sea water over the gills. Receptive fields were mapped on a white screen which the animal viewed through the wall of a clear plexiglass tank.

The most common response found in the optic lobe was a transient on response to stimulation with a spot of light in the appropriate part of the field of view. A large spot of light gave less response than a small spot of light. The center of the receptive field varied in size from 20° to less than 3°. In contrast, the responses obtained from the optic nerve were always on-responses sustained for the duration of illumination, with no evidence of an inhibitory surround to the receptive field. A few off-center units were found in the optic lobe, with receptive fields of 10°–20°. One unit responded to movement of light in the rostral-caudal direction, but not in the caudal-rostral direction. Most of the receptive fields were near the horizontal and less than 45° from the optic axis, due to limitations on placement of the electrode.

Cytochemical localization of materials within the sperm of Libinia emarginata. Raymond G. Fenwick, Jr. and Gertrude W. Hinsch.

Cytochemical examinations of the testis and mature sperm of *Libinia emarginata* have demonstrated patterns of localization of several substances. The Feulgen reaction was used to identitfy DNA; alkaline fast green and bromphenol blue for somatic histones and basic proteins; alkaline fast green and bromphenol blue with previous deamination or acetylation for arginine-rich basic proteins, lysine-rich basic proteins, and protamines; and dimethylaminobenzaldehyde-nitrite for tryptophan. The nuclei of somatic cells and spermatocytes contain adult-type somatic histones. The nuclei of maturing spermatids in the testis do not give positive reactions for histones. However, nuclei of mature sperm taken from the seminal receptacle of a female crab do contain protamines, this in contrast to the results of other investigators who used sperm from the testis or spermatophores. This suggests that spermatid maturation may be completed in the spermatophore or seminal receptacle and that the nucleus of a mature decapod sperm is similar to that of mature flagellate sperm in possessing basic protein.

Within the acrosome of the maturing spermatid, the apical cap contains an arginine-rich, nonprotamine basic protein and the acrosomal rays and thickened ring contain a lysine-rich basic protein. Tests have demonstrated that if these are histones which have migrated from the nucleus, as has been postulated, they are associated with nonhistone proteins. The acrosomal rays and thickened ring of the mature sperm appear to contain an arginine-rich basic protein in addition to the lysine-rich basic protein of the spermatid.

This study was performed in the Fertilization and Gamete Physiology Training Program at the Marine Biological Laboratory (NIH grant T1-HD-26-08).

Gel filtration of chromatophorotropins from eyestalk extracts of the fiddler crab, Uca pugilator, *and the prawn,* Palaemonetes vulgaris. MILTON FINGERMAN AND CLELMER K. BARTELL.

Aqueous extracts of eyestalks from the fiddler crab, *Uca pugilator,* were filtered through a Bio-Gel P-6 column (71 × 1.5 cm), eluted with distilled water. Fractions were adjusted to isosmotic saline concentrations and assayed for black, red and yellow pigment-dispersing activities, for white pigment-concentrating activity in eyestalkless crabs, for black, red, yellow pigment-concentrating and white pigment-dispersing activities in crabs from Panacea, Florida, that were adapted to a black background, and, finally, for red pigment-concentration in eyestalkless prawns and red pigment dispersion in white background-adapted prawns, *Palaemonetes vulgaris,* from Woods Hole, Massachusetts. Aqueous extracts of prawn eyestalks were also filtered through the column and assayed for black, red, and white pigment dispersion in crabs and for red pigment concentration in prawns. Neither black pigment concentration in crabs nor red pigment dispersion in prawns was detected in assaying the fractionated extracts. The mobility values for the pigment-dispersing factors were about 0.6 and for the concentrating factors about 0.28. The activity peaks departed from a bell-shaped normal distribution, suggesting that more than one component of each activity may be present. These several activating substances from eyestalks were separated clearly into pigment-dispersing and concentrating components. However, the dispersing activities for the different chromatophores did not separate from each other. Likewise, the concentrating activities did not separate from each other.

Aqueous suspensions of the residue from alcohol-soluble fractions of both crab and prawn eyestalks were filtered through the column. Both extracts yielded two peaks of red and black pigment-dispersing activity in crabs. Other activities were not examined. The first peak eluted in the void volume and earlier results support the hypothesis that this active component is associated with a lipoprotein fraction. The second peak had a mobility of about 0.6 in both extracts and is thought to be similar to the active material in aqueous extracts.

Supported by Grant GB-7595X from the National Science Foundation.

The control of the initiation of asexual reproduction in the tunicate Amaroucium constellatum. G. FREEMAN.

After the tadpole of *Amaroucium* undergoes metamorphosis the oozooid increases in size at a logarithmic rate for about two weeks, then size increase stops and within two days the animal reproduces asexually by strobilating. During this process a series of constrictions appear along the body column which divide the animal into 5–12 segments; each segment then differentiates to form a new zooid.

If the branchial basket is removed from an oozooid which is between one and six days old, the branchial basket is regenerated, the animal grows for about two weeks, and then strobilates. If the same operation is done on an animal which is seven or more days old, the animal always strobilates within one day after the operation. If the postabdomen or the top third of the branchial basket are removed at any time during the growth of the oozooid, in almost every case the operated animals strobilate at the same time that the unoperated controls strobilate.

By using grafting techniques it is possible to produce zooids with two functional branchial baskets (siamese twin zooids). These animals strobilate at the same time that normal animals of the same age strobilate. If one removes both branchial baskets from a siamese twin zooid which is seven or more days old, the animal always strobilates within one day after the operation. If one branchial basket is removed from a siamese twin zooid which is seven or more days old, the animal does not strobilate immediately. If the branchial basket which has been removed does not regenerate the operated zooid strobilates at about the same time as unoperated normal animals which are the same age; if the branchial basket regenerates the zooid grows for about two weeks and then strobilates.

These experiments suggest that there is an organ in the branchial basket which controls the timing of strobilization.

Supported by a grant from the National Science Foundation.

Microspectrophotometry of rhabdoms of the spider crab Libinia. Timothy H. Goldsmith and Davis Hays.

Isolated dark-adapted rhabdoms from the spider crab *Libinia emarginata* were examined by microspectrophotometry to determine the visual pigments present and their light-sensitive characteristics. The rhabdoms contain a single pigment with $\lambda_{max} = 493$ nm. Upon one minute irradiation with bright orange light this pigment forms a light-stable photoproduct with nearly the same λ_{max} as the parent pigment but with slightly greater absorption to the long wavelength side of the absorption peak. On exposure to orange or yellow light in the presence of 5% glutaraldehyde, however, the pigment of *Libinia* rhabdoms bleaches slowly.

The photosensitive pigment of properly oriented, transversely illuminated rhabdoms shows isotropic and dichroic regions, corresponding to layers of the rhabdom in which the microvilli are, respectively, parallel and perpendicular to the direction of propagation of the measuring beam. The maximum dichroic ratio is about 2, with most absorption when the plane of polarization is parallel to the microvillar axes.

This work was supported by U.S.P.H.S. grant NB-03333 to Yale University.

Genetic studies on marine species of the Phylum Ectoprocta. James L. Gooch and Thomas J. M. Schopf.

Polyacrylamide gel electrophoresis of esterases, malate dehydrogenase, and "leucine" aminopeptidase revealed genetic variants at single loci. Gene and genotype frequencies were determined at 9 loci and the amount of genetic variability was estimated for *Schizoporella unicornis* and 3 species of *Bugula* (*B. stolonifera, B. turrita,* and *B. simplex*). The following conclusions were made.

1. A large amount of genetic polymorphism (an average of 48.5 per cent for 4 species, with an average of 8.25 loci sampled per species) exists in marine ectoprocts. This indicates an amount of genetic variability fully comparable to that of terrestrial animals.

2. Comparisons of genotype frequencies in three species were made with values predicted by Hardy-Weinberg equilibria, with the result that each species proved to be chiefly outbreeding.

3. Significant differences in gene frequencies exist on the order of 10–20 kilometers in *S. unicornis*. This indicates that the local population (deme) size occupies an area smaller than this.

4. Major and consistent differences in electrophoretic mobility of the same type of protein occurred in 12 ectoproct species, thus showing the taxonomic applicability of this approach.

Supported in part by NSF grant GB-7325 to Thomas J. M. Schopf.

Fine structure of the fiddler crab epidermis. JONATHAN P. GREEN AND MARY ROSE NEFF.

The crustacean epidermis contains cellular elements involved in the deposition of the exoskeleton and in color change. It consists of secretory cells next to the exoskeleton, a spongy connective tissue layer, and a basement membrane. The epidermis underlying the carapace of the fiddler crab, *Uca pugnax,* additionally has a reticular layer of cells next to the basement membrane. Secretory cells are richly supplied with mitochondria, endoplasmic reticulum, and Golgi. Stellate black, red, yellow, and white pigment cells weave between them but melanophores alone are found in the reticular layer. No tight junctions involving pigment cells were demonstrable. Pigment granules vary in size and shape: Round to ovoid melanosomes have a mean diameter of 0.55 μ; round erythrophore granules, 0.45 μ; round xanthophore granules, 1.5 μ; and polygonal white granules, 0.5×0.15 μ. Pigment cell processes are oriented parallel to the plane of the epidermis, providing maximum color variation upon pigment migration.

Electrotonic coupling could not be demonstrated between fiddler crab melanophores (Freeman and Green 1969, unpublished) in contrast to *Palaemonetes* erythrophores (Freeman *et al.*, 1968, *Comp. Biochem. Physiol.*, **26**: 1015–1030).

Freeman *et al.,* (1968) hold that red pigment-concentrating hormone leads to hyperpolarization of the erythrophore membrane, not because of changes in ionic permeability, but because of volume changes ($\geqslant 40\%$). Electron microscopic observations of melanophore processes show that they expand and deform, and collapse as the melanosomes migrate. No estimate of the number or extent of melanophore processes is available but a 40% volume change during pigment migration is not unreasonable.

Microtubules are absent in fiddler crab melanophores, suggesting that a "railroad track" mechanism similar to that proposed for the movement of *Fundulus* melanosomes is not applicable. Vinblastin ($5 \times 10^{-5} M$) coupled with melanophore-expanding hormone did not effect any change in the rate of melanosome migration. The hypothesized lack of involvement of microtubules in fiddler crab melanosome migration, the lack of tight junctions, and the lack of electrotonic coupling between melanophores may be related to the fact that these cells are hormonally rather than neurally driven.

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The effect of inhibitors of proteolysis on sea urchin embryogenesis. ALBERT GROSSMAN, JAMES ELSTER, WALTER TROLL AND MILTON LEVY.

Proteolytic activity has been found to be associated with the "membrane" fraction of sea urchin (*Arbacia punctulata*) egg extracts. The "membrane" fraction is defined as the residue remaining after washing and centrifuging sonicated egg extracts at $1000 \times g$ six times. The material is capable of hydrolyzing tosyl-arginine methyl ester (TAME) and acetyl-lysine methyl ester at approximately equal rates. This is unlike trypsin, which has considerably greater preference for TAME. Solubilization of the proteolytic activity was accomplished by sonicating the washed "membrane" in 1% detergent (cutscum, Fisher). The specific activity was 60 mμmoles/hr per mg protein with TAME as a substrate. Activity was proportional to enzyme concentration and was heat labile. Polyarginine also appeared to be a substrate.

The observation that "membrane" proteolytic activity increases 5-fold during early embryogenesis supports the suggestion made by Monroy and his co-workers that the increase in protein synthesis after fertilization may be associated with proteolytic activity. Presumably, such activity unmasks the latent protein synthesizing capacity of the egg. Based on this assumption, inhibitors of proteolysis should prevent development. Given 10 minutes prior to fertilization at concentrations of 1–10 mM, the inhibitors tosyl-phenylalanyl-chloromethyl ketone and tosyl-lysyl-chloromethyl ketone are 97% effective in preventing the first division. At the same time these substances markedly depress C-14 lysine and C-14 phenylalanine incorporation into protein. Whether the effects produced by these inhibitors are due solely to their capacity to inhibit proteolysis remains to be determined.

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Comparative biochemistry of calcium-activated photoproteins from the ctenophore, Mnemiopsis *and the coelenterates* Aequorea, Obelia, Pelagia *and* Renilla. J. W. HASTINGS AND J. G. MORIN.

Two bioluminescent systems which have up to now appeared quite different biochemically have been described from different classes of coelenterates. We believe that these two systems, characterized especially in *Renilla* (Cormier) and *Aequorea* (Shimomura *et al.*), are actually parts of a unitary biochemical mechanism common to all classes. In *Renilla* the reaction was known to involve at least two enzymatic steps: luciferin activation followed by its oxidation with O_2, calcium being required. From *Aequorea* a protein was isolated which gives light simply upon adding calcium, without O_2. We have isolated a similar calcium-activated protein from *Renilla,* indicating that all the coelenterate systems are basically similar. The calcium-activated protein is postulated to be a relatively stable enzyme intermediate, probably a peroxide formed in reactions such as those demonstrated in *Renilla.* Control of flashing could thus be mediated by calcium mobilization.

Within the framework of the common calcium-activated system distinctive differences have been observed among the five genera. In some (*Obelia, Aequorea, Renilla*) there is a shift in color from the blue of the isolated *in vitro* reaction to the green (505 nm) of the *in vivo* luminescence. This is attributed to energy transfer from the luminescent system to a green fluorescent protein associated with the photogenic granules. Its *in vivo* fluorescence is diagnostic of this shift and serves to identify the granules. There are also substantial differences in the color of the different *in vitro* calcium-activated proteins (460–485 nm), indicating that each is chemically distinct. They also exhibit differences in kinetic parameters and pH-activity profiles. The first order rate constants at 25° for the decay of the *in vivo* flash range from 10 (*Aequorea*) to 50 sec^{-1}(*Obelia*) and are much faster than the *in vitro* reactions, which range from about 0.2 (*Renilla*) to 4 sec^{-1} (*Obelia*). The fact that luminescence occurs as a flash in many forms, whereas in some (*Pelagia*) it is more like a glowing secretion, indicates that there are different modes of control.

Hyperpolarizing inactivation and activation in crayfish neuron soma. HIDEO HAYASHI.

Neurons of the crayfish *Procambarus clarkii* (soma of the median giant fiber or large motoneurons in abdominal ganglia) develop hyperpolarizing responses similar to those of lobster muscle fibers. In the normal saline (5.4 m*M* KCl) during the application of a constant inward current the membrane undergoes hyperpolarization to a large peak that is followed by a plateau. The large hyperpolarization (200–250 mv from the resting potential) results from a decrease in conductance (inactivation) by up to 3 times. The plateau hyperpolarization was 75 to 120 mv from the resting potential and usually decreased markedly with increasing inward currents. On terminating the current the membrane depolarized by about 1[illegible] mv. During clamping with low voltage steps (< 60 mv) only inactivation was observed. With higher voltages the inactivation was brief and the current then increased greatly denoting large activation. Slowly increasing ramp voltage clamps emphasized the inactivation process while rapidly increasing ramp voltages induced only activation. Both processes persisted after the ramp was terminated. Changes in external K or Cl had little effect on the conductance or emf of the plateau phase. Increase of pH from 7.4 to 9.8 or application of TTX (4×10^{-7} g/ml) decreased the activation and reduced or abolished the after depolarization. The after depolarization was diminished on removing Na but the conductance during the plateau was almost unchanged.

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Microtubules in spermatozoa of some turbellarian flatworms. CATHERINE HENLEY AND DONALD P. COSTELLO.

At least two patterns characterize the arrangement of microtubules in spermatozoa of flatworms, the 9 + 0 and the "9 + 1." The 9 + 0 arrangement is found in motile spermatozoa of *Childia* and of *Polychoerus carmelensis,* as reported by us last year, and has also been found to be present in spermatozoa of two more acoels, *Anaperus* and a Nantucket species of *Polychoerus.* As seen by light microscopy the spermatozoa of both these forms are motile and very long and slender (*ca.* 400 μ in length), with two axonemes incorporated into a double undulating membrane. Spermatids of the Nantucket species of *Polychoerus,* like those of *P. carmelensis,* have a large array of cortical singlet microtubules, in addition to the doublets of the axial units, as seen in sectioned material by electron microscopy. Both singlets and doublets are bound together at the posterior end by a single caplike structure, in preparations negatively stained with phosphotungstic acid; presumably this is the case in sectioned material also, although we have not yet demonstrated it. No evidence has been found to date of a comparable binding structure in spermatids of *Anaperus,* but in view of the striking similarity in morphology of the two sperm-types, it very likely does occur there as well.

The living spermatozoon of the triclad *Bdelloura* has a motile body about 100 μ long, and two very long free flagella, *ca.* 200 μ in length. The flagella have been shown by Silveira and Porter to have a "9 + 1" pattern of microtubules in sectioned material. We have confirmed this using negative staining, and have demonstrated a striking structure of the central core which is much like that found in free flagella of spermatozoa from another triclad, *Dugesia,* and a rhabdocoel, *Mesostoma.* In all three, there are two hollow structures wound in a double helix around a solid innermost element, in negatively stained material.

We believe the differences in patterns of microtubules are closely associated with differences in motility and morphology of flatworm spermatozoa, and are continuing a systematic survey of the group.

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Characteristics of the isolated hyaline layer. ELENA CITKOWITZ HINEGARDNER.

A method has been developed for isolating intact hyaline layers from sea urchin embryos at the blastula and gastrula stage. Embryos are suspended in a solution containing 4 M NaCl, 0.2 M $MgCl_2$ and 0.01 M Tris, pH 8 and homogenized in a Dounce homogenizer. The hyaline layers are partially purified with nylon monofilament bolting cloth and finally isolated on a discontinuous surcose gradient. Extremely pure preparations are obtained in yields of 80–90%. The isolation technique is used most extensively on *S. purpuratus* and has also been successfully applied to the sand dollar (*E. parma*), the starfish (*A. forbesi*), and the urchins, *A. punctulata* and *L. pictus.*

The hyaline layer of the blastula is a transparent sac. The gastrula hyaline layer, however, has a projection which lines the archenteron on the intact embryo. The projection is present in hyaline layers obtained from all stages of gastrulation, the length of the projection corresponding to the degree of invagination. At a given stage of development, the hyaline layers of all five species show the same basic structure.

Amino acid analysis of hydrolyzed *S. purpuratus* blastula and gastrula hyaline layers shows both to be high in proline and threonine and low in histidine, lysine, arginine and methionine. Protein content determined by the Lowry method is equal to the dry weight. Staining with alcian blue indicates that carbohydrate is also present. When dialyzed against a solution of 0.1 M NaCl and 0.01 M Tris, pH 9, the hyaline layer dissolves almost completely. Analytical ultracentrafugation of blastula hyaline layers in this solvent shows a single hypersharp peak unless the material is extremely dilute. The uncorrected sedimentation coefficient at a concentration of 0.02% at pH 9 is 5.6.

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Schwann cells of lobster walking limb nerves; A morphological and cytochemical study. ERIC HOLTZMAN, ALAN R. FREEMAN AND LEONARD A. KASHNER.

The Schwann cells associated with giant axons in walking limbs contain elaborate network-like systems of anastomosing tubules that are seen every few microns along the length of the axon in favorable sections. The tubules are 20–40 millimicrons in diameter and are delimited by "unit" membranes. The networks are connected to the Schwann cell plasma membrane both at the axon Schwann cell space and at the opposite surface where the Schwann cell faces connective tissue. Often the tubules are also connected to other channels that penetrate the Schwann cell sheath such as the flattened spaces found where adjacent Schwann cells abut.

When isolated nerves are soaked in Ringers solution containing horseradish peroxidase and then are incubated for demonstration of peroxidase activity, reaction product is found in the axon Schwann cell space. The anastomosing tubules also contain reaction product; this indicates that the tubules are accessible to extracellular materials as might be expected from their connection to the plasma membrane.

In light of the frequency and dimensions of the anastomosing tubules these findings suggest that the networks are a major pathway through the Schwann cell sheath.

Peroxidase also accumulates in coated pinocytosis vesicles in the Schwann cells. Such vesicles are sometimes seen attached to the tubules as in forming from them. This pinocytosis activity may reflect exchanges between Schwann cell and extra-cellular fluid; the large surface area of the networks would facilitate such exchanges.

In preparations incubated with Karnovsky's cholinesterase methods, with either acetyl- or butyryl-thiocholine used as substrate, reaction product is found among the cells immediately outside the Schwann cell sheath. With butyrylthiocholine we have also observed reaction product in the sheath; it is found in the axon-Schwann cell space and in the networks of tubules.

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Some effects of amphotericin B on isolated lobster walking limb axons. RONALD W. HOLZ AND ALAN R. FREEMAN.

Amphotericin B, a polyene antibiotic of known structure, causes selective permeability changes in Mueller and Rudin thin lipid films. It is of interest to determine its effects on an excitable biological membrane.

Freeman *et al.* have shown that the fast closer axon proper of the lobster walking limb obeys the van't Hoff relation for small osmotic challenges. It is thus possible to measure osmotic water permeability changes induced in this axon by amphotericin B. It is found that the osmotic water permeability, P_f, in the presence of $2 \times 10^{-5} M$ amphotericin B equals 3.7×10^{-3} cm/s (standard deviation 28%) as compared to 1.0×10^{-3} cm/s (standard deviation 24%) for the control. Hence, amphotericin B increases the osmotic water permeability.

Amphotericin B also blocks the propagated action potential as externally recorded. The effect is reversible if the preparation is washed with amphotericin free solution at 33° C, but seems irreversible if it is washed with amphotericin free solution at 23° C.

These effects, the increase in water permeability and the temperature effect on reversibility of the action potential blockade, are consistent with the actions of the antibiotic on thin lipid films. In addition it should be pointed out that as far as is known, amphotericin B will interact with biological and artificial membranes only if they contain sterol. This strongly suggests that the fast closer axon membrane of the lobster walking limb also contains sterol, a determination that is difficult to make directly.

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Arbacia punctulata *blastulae: structure analysis with the surface replica method.* SADAYUKI INOUÉ, GILLES H. COUSINEAU AND PAUL L. KRUPA.

Ultrastructural observations of the surface of *Arbacia punctulata* blastulae were made using the pre-shadowed carbon replica method. Blastulae were freeze-dried in a minimum amount of sea water 6 hours after fertilization. Dried blastulae were floated on the surface of a small volume of 15% cellulose nitrate/acetone solution on a glass slide. Drying of the cellulose nitrate left the lower half of each blastula embedded and the upper hemisphere exposed on a solidified film. Platinum shadowing and carbon coating were done in the usual manner, followed by the application of a layer of paraffin. Then the dried cellulose nitrate was dissolved away in acetone and the blastulae were decomposed in 16 N H_2SO_4 for about 10 hours at 26° C. Finally, after the paraffin was dissolved in benzene, the surface replica of the blastulae was examined in the electron microscope. The replicated surface was found to be relatively smooth, with randomly distributed, irregularly bent fibrils measuring about 120 Å in width. Except for this uniformity in diameter of the fibrils, no regular substructural pattern has been discernible thus far. However, crystal-like, elongated particles (about 0.3 μ wide and 0.8 μ long) were occasionally seen projecting from the matted network of fibrils.

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Selection and isolation of luminescent bacteria from nature with new features. ALEX KEYNAN AND J. W. HASTINGS.

Luminescent bacteria isolated from nature are usually bright (10^3–10^4 photons sec^{-1} at optimal intensity). Since they are selected in the first place for their light emission, it is not surprising that there is very little knowledge concerning the occurrence and properties of dim strains in nature. This is of interest because both mutations to dim strains and reversions to brights occur readily in the laboratory. It would be important to know whether this apparent lability is only a laboratory phenomenon or whether it occurs also in nature and is of ecological significance with regard to the control of the property of light emission.

An additional question relates to the uniformity of luminescent strains in nature. In the past they have usually been selected from the surface of newly decomposing fish, an enrichment situation in which peptones are the nitrogen source. It seemed of importance to determine whether luminous bacteria which are capable of growth on nitrate as the sole nitrogen source occur in nature. If so, it would indicate that luminous bacteria are not confined to a single or restricted ecological situation.

Bacteria were isolated at 20° C from several sources: the surface of a newly decomposing fish, marine mud, and by rinsing sea water on agar plates. In the latter case, using nutrient agar plates, there were about 5 luminescent colonies among a total of 300; with nitrate as the sole nitrogen source, there were many fewer colonies (10–100), but surprisingly, luminous ones were almost as frequent. Their growth, judged by colony appearance, was relatively slow on nitrate (3 days) compared to a peptone containing medium (1 day), but light emission is good on both media. The bacteria have remained stable through several transfers. Screening for dim strains has been more difficult but seven such isolates have been obtained. However, they appear not to be so dim as those which occur in the laboratory. It is believed that improved techniques will facilitate obtaining even dimmer strains, if they occur.

Ionic effects on the Strophanthidin inhibition of the sodium pump in squid giant axon. JUAN I. KORENBROT.

The sodium pump in the squid giant axon depends on metabolic energy for its proper function and is sensitive to the inhibitory action of externally applied cardiac glycosides, such as ouabain and Strophanthidin. The inhibitory action of the glycosides is dependent upon the metabolic state of the axon and the ionic composition of the external medium.

The activity of the sodium extruding pump was followed by measuring the Na^{22} efflux from carefully cleaned giant axons of *Loligo pealei* loaded by microinjection. Samples were collected at preset time intervals of 15 to 120 seconds for periods of up to two hours. The time to half inhibition was measured as a function of Strophanthidin concentration at 19–20° C. For Strophanthidin concentrations of $10^{-8}\,M$, $10^{-7}\,M$ and $10^{-6}\,M$ these times were 15.4 min, 10.2 min and 1.5 min, respectively. ASW solutions of $10^{-7}\,M$ Strophanthidin were prepared with various Na^+ and K^+ concentrations. It was found that for a given Na^+

concentration, 430 m*M*, the percent of maximal inhibition decreased with increasing K^+ concentrations, 60% at 10 m*M*, 33% at 40 m*M*. For a given K^+ concentration it increased with increasing Na^+ 36% at 107 m*M*, 60% at 430 m*M*. More important, the percentages of inhibition were similar as long as the Na^+/K^+ ratio was constant, $61.0 \pm 1.2\%$ for 43/1, $34.7 \pm 1.2\%$ for 10.7/1.

Most of the available models for the sodium pump propose the existence in the membrane outside of two states with different binding affinities for Na^+ and K^+. Further, one of the states, Na^+-binding, changes into a K^+-binding state during normal activity. It is proposed that this change is prevented by the action of Strophanthidin, thus inhibiting the translocation of cations across the membrane.

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Electron microscopy of colloid absorption by the surface of a platyhelminth parasite. PAUL L. KRUPA, GILLES H. COUSINEAU, SADAUKI INOUÉ AND KARL WITTMAN.

Thorium dioxide (Thorotrast) was used to study particle uptake by the tegument of the trematode, *Cryptocotyle lingua*. Rediae and intramolluscan cercariae were dissected from their snail host, washed clean of host debris, and immersed in a solution of 25 mg Thorotrast per 1.0 ml MBL Formula sea water at 20° C for 15, 30, 60, 120, and 240 minutes. Following each period of incubation, the parasites were fixed in glutaraldehyde, postosmicated, and embedded in Epon for electron microscopy. Controls were placed either in Thorotrast premixed with glutaraldehyde, or in glutaraldehyde followed by a 1-hour immersion in Thorotrast solution.

Phagocytosis of the thorium dioxide by the apical cytoplasm of rediae occurs during all periods of incubation. Colloid particles adhere to the surface of the parasite in a thin, glycocalyx-like layer. After a 15-minute incubation, colloid appears in phagocytic surface invaginations and membrane-bounded vacuoles. Randomly scattered colloid particles are also found in the cytoplasm of the tegument and surface flaps. In parasites incubated for 30 minutes or longer, single particles or aggregates of particles occur in the basement lamina, mitochondria, muscle cells, fat globules, and intercellular spaces of the body wall, as well as in the brood chamber containing the cercarial embryos. Although the surface flaps often sequester thorium dioxide particles for subsequent incorporation into vacuoles, sometimes the flaps themselves are also entrapped along with the colloid by long cytoplasmic extensions from the tegument. Preliminary observations suggest that thorium dioxide is also absorbed by the surface of intraredial cercariae and cercariae developing in snail tissue following escape from rediae.

Since Thorotrast is stabilized with dextrin, a polysaccharide which probably remains adsorbed to the colloid particle, the absorptive processes visualized in this study may also reflect activities of these and other parasites during uptake of exogenous carbohydrate.

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Ionic effects on the relationship between pre- and postsynaptic potentials. KIYOSHI KUSANO.

Effects of various ions on the synaptic transfer characteristics were studied, using untreated or presynaptically TEA-injected squid giant synapses, in the presence of 10^{-6} g/ml tetrodotoxin. Employing 10 msec presynaptic pulses average values obtained from 19 untreated preparations showed that (1) presynaptic depolarization for producing 1 mV of PSP was 25 mV, (2) presynaptic depolarization for a tenfold change of PSP was 12.5 mV, (3) maximum amplitude of PSP was 36 mV, and (4) corresponding presynaptic depolarization was 65.5 mV. In TEA-injected preparations the values for (1), (2) and (4) were lowered to some extent. The efficiency of the synaptic transfer functions was augmented in general by increasing $[Ca^{++}]_o$ or by reducing $[Mg^{++}]_o$. Opposite results were found by lowering either $[Na^+]_o$ or $[Ca^{++}]_o$, or by increasing $[K^+]_o$ or $[Mg^{++}]_o$. When $[K^+]_o$ was increased up to fourfold amplitude of the minimal presynaptic depolarization (MPD) for a detectable size of PSP was sometimes reduced (*e.g.* from 26 mV to 16 mV). Removal of $[K^+]_o$ showed small increase of MPD in some preparations but in general it had no effect. In a $[Ca^{++}]_o$-free medium the presynaptically applied depolarization failed to produce both On- and Off-PSP's. The minimal level of presynaptic depolarization which suppressed On-PSP completely averaged 106 mV inside positive (ranging between 54–200 mV from 8 preparations). One factor in the spread is the small space constant of the terminal (between 450 μ to nearly 1 mm). The potential level was shifted more negative on lowering $[Ca^{++}]_o$ and was shifted more positive in high $[Ca^{++}]_o$-media. However, altering the $[Na^+]_o$ did not change level. Alteration of $[Mg^{++}]_o$ also did not affect the level significantly in two out of three preparations. Under these ionic circumstances the maximum amplitude of On-PSP as well as Off-PSP was markedly changed but the presynaptic depolarization to evoke a maximal On-PSP was essentially unchanged. Although the data are still qualitative in nature, they appear to support the "Ca hypothesis" for the transmitter release and its shut-off mechanism.

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Chromosome preparations in Artemia salina. PAUL H. LAMARCHE, JOSEPH D. CASSIDY, O.P. AND DANIEL S. GROSCH.

Ploidy differences have been reported in this cosmopolitan genus. Genetic studies are in conflict regarding reproductive isolation among races of the brine shrimp. Detailed chromosomal analyses utilizing filtration and washing with crustacean Ringers solution, 0.125% trypsinization, controlled (0.5–1.0% NaCl) hypotonic shock, and DNA-specific cytochemistry were applied to free-swimming nauplii and differentiating gonadal tissue of adult males. These preparations were examined by bright field, phase contrast and scanning electron microscopy. The condensed DNA in the mitotic and meiotic cells of two races derived from San Francisco (stock #3) and Utah (Great Salt Lake) were of unusual configuration. Plate-like chromosomal units having thread-like, filamentous extensions were observed. Numerous microchromosomes were connected to the larger chromatin bodies by the filamentous threads. Resolution and contrast of these m-chromosomes, enhanced by the scanning electron microscope, detailed their ultrastructure in bas-relief. These techniques provided slides covered with hundreds of well-spread metaphase figures for comprehensive cytogenetic comparison of both races of *Artemia.*

Centromeric variation in some chromosome bodies included meta, acro, and submedian types. Cellular mosaicism was detected in confirmation of earlier studies. These methods may facilitate the analysis of speciation within this genus and may prove useful in the cytogenetics of other invertebrates.

Changes in light scattering during synaptic activity in the electric organ of the skate, Raia erinacea. D. LANDOWNE AND L. B. COHEN.

In an attempt to obtain information about the structural changes that occur during synaptic transmission, we have measured changes in light scattering following stimulation of the electric organ from the skate.

Sections of the electric organ were illuminated by light perpendicular to the long axis of the electric organ. The light scattered at 90° to both the incident light and the long axis was measured. The organ was stimulated by methods similar to those of L. G. Brock and R. M. Eccles.

Following stimulation, the intensity of the light scattered at 90° first transiently increased. This transient increase was followed by a longer lasting decrease in scattering. No optical change was observed before the beginning of the postsynaptic potential. The initial increase reached a peak at about 10 msec after the stimulus, and the increase averaged 2.2×10^{-5} of the resting scattering. The subsequent decrease in scattering persisted for more than 100 msec. The average size of the decrease was 2.6×10^{-5} of the resting scattering.

Both scattering changes were blocked when the Ringer's solution contained 10 μM curare. Similarly, both changes were blocked when the Ringer's solution contained 200 μM acetylcholine plus 20 μM eserine. These changes in bathing medium block the postsynaptic potential, presumably without drastically altering the presynaptic events. With this assumption, these experiments showed that the light scattering changes were caused by structural events related to the action of the transmitter substance on the postsynaptic cells and were not related to the processes of release of transmitter. We do not know which of the events occurring during the postsynaptic potential are giving rise to the scattering changes.

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The effect of polyols on gamma crystallin of the dogfish lens. SIDNEY LERMAN AND HOWARD A. JONES.

Sugar alcohols such as sorbitol, xylitol and dulicitol accumulate quite rapidly in the experimental sugar cataracts. Aside from their osmotic role in the pathogenesis of sugar cataracts it is possible that these sugar alcohols, in high concentrations, might affect the native protein molecules as well. An aqueous solution of purified dogfish gamma crystallin (125 μg/ml) in 0.01 M phosphate buffer at pH 7.5 and 8.5 was employed for this study. The gamma crystallin was purified by means of DEAE cellulose column chromatography followed by starch block electrophoresis. The effects of 2.5% and 10% concentrations of sorbitol, xylitol and urea as well as a combination of urea + 2.5% and 10% solutions of the sugar alcohols were investigated in the Cary 14 recording spectrophotometer and in the Cary 60 spectropolarimeter. The studies were performed at 25° C and at 65° C. Aqueous solutions of these sugar alcohols appeared to exert a protective effect on gamma crystallin with respect to denaturation and the solubility of gamma crystallin also appeared to be enhanced. ORD and CD studies on purified gamma crystallin revealed that it is a highly structured protein consisting mainly of beta helix with some alpha helical conformation as well. Neither urea or the sugar alcohols exerted any effect on gamma crystallin with respect to its optical rotatory dispersion and circular dichroism. It is postulated that the effects of the sugar alcohols could be attributed to a strengthening of the hydrophobic bonds in the protein molecule induced by the surrounding medium and to a decrease of the hydrogen bond rupturing capacity of the solvent.

This work was supported by a Medicāl Research Council Grant MA 3208.

How divalent cations and temperature affect transmitter release at the giant synapse in the squid stellate ganglion. HENRY A. LESTER.

1. Intracellular microelectrode recording techniques were used to study stellate ganglia bathed in sea water containing subnormal calcium concentrations ([Ca^{++}]). When [Ca^{++}] was increased, the postsynaptic potential (PSP) took from 20 min to 1.5 hr to equilibrate to a new value; this time was approximately doubled for decreases in [Ca^{++}].

A plot of PSP amplitude versus [Ca^{++}] gave a straight line on double-logarithmic coordinates. For four such experiments, the average slope of this line was 3.6 ± 0.3. This relationship suggests a molecular model in which three or four calcium ions must bind in unison at a rate-limiting step in the process of transmitter release.

2. In preliminary experiments with sea water containing normal [Ca^{++}] and varying [Mg^{++}], postsynaptic impulses were blocked by hyperpolarizing the postsynaptic terminal. The inhibition of PSP amplitude by magnesium was too strong to fit a simple competitive model in which a single magnesium ion can inhibit the rate-limiting step by binding to one of the calcium sites.

It was not possible to monitor the presynaptic impulse intracellularly during the entire run of a [Ca^{++}] or [Mg^{++}] series. However, fragmentary results indicate that the presynaptic action potential was unchanged in amplitude under the conditions studied.

3. When the postsynaptic terminal was hyperpolarized with applied current, the PSP increased linearly with membrane potential. Extrapolation of this relationship to zero PSP gave a transmembrane reversal potential of zero. This linearity may be exploited to compare PSP's measured at different values of membrane hyperpolarization.

4. With microelectrodes inserted in the presynaptic and postsynaptic terminals, synaptic delay was studied as a function of temperature. From 9° to 24°, the Q_{10} of the synaptic delay was 2.5.

This work was conducted during the tenure of an NIH predoctoral fellowship with the support of Research Grant B864 from the National Institute of Neurological Diseases and Blindness and of Research Grant GB-6540X from the National Science Foundation.

Comparative immunochemical studies on alpha crystallin in the ocular lens. PANKAJ MEHTA AND SIDNEY LERMAN.

Alpha crystallins derived from the dogfish, rat, chicken, rabbit, sheep, bovine, monkey, and human lens, were purified by isolectic precipitation on gel filtration. Each alpha crystallin was shown to be homogeneous by immunological criteria. The quantitative complement fixation test was employed to study the immunological cross reactions of the alpha crystallins. Antisera specific for bovine alpha crystallin were used in these experiments. The results of these studies indicate that while immunodiffusion techniques fail to detect most of the antigenic differences between the alpha crystallins, the quantitative complement fixation test distinguished between dogfish alpha crystallin (which showed no fixation) and the other alpha crystallins. The bovine, sheep, and rabbit alpha crystallins appear to be identical, as judged by the quantitative complement fixation technique, as are the human and monkey alpha crystallins which showed a similar but significantly lower degree of fixation. Rat alpha crystallin showed approximately 10–15% fixation.

All the foregoing alpha crystallin demonstrated three bands in starch gel electrophoresis, pH 3.0, in 8 *M* urea but there were differences in mobility.

This work was supported by a Medical Research Council Grant MA 3208.

Identification of Woods Hole species of Tubularia. RICHARD L. MILLER.

The following are summary descriptions of the three common species of the hydroid *Tubularia* found in the Woods Hole region. Investigators using *Tubularia* should be certain that the organisms are in optimal condition before attempting identification. The seasons given are based on Supply Department collections.

Tubularia spectabilis (Agassiz) is found on the Fisheries and WHOI docks and the spindle from October to early July and in Cape Cod Canal to late July. It has long straight stems and branches exclusively basally. The tentacle numbers average 20–25 in both proximal and distal sets. The male gonophore bears four very short, rounded protuberances at its apex whereas the female bears 3–5 moderately long, usually clumped tentacles.

Tubularia crocea (Agassiz) is found in New Bedford Harbor and sometimes on the WHOI dock from late July to early September. The stems are like those of *T. spectabilis* except for the presence of internal longitudinal striations and the sporadic occurrence of groups of weak annulations. The tentacle numbers are the same as *T. spectabilis.* The male gonophore has no tentacles. The female gonophore bears 8–10 flat, bladelike protrusions (tentacles) arranged radially around the apex. The blastostyle protrudes through an opening in the apex.

Tubularia larynx Ellis and Solander is found in the Cape Cod Canal from late June until early August and sometimes throughout the summer. The stems are partially or continuously annulated, most heavily at the branch points. The over-all stem length is shorter than the other species but may reach 3–4 inches in height due to branching. Branching may be profuse, to within one-half inch of the polyp, or sparse, but does occur above the base of the colony. The numbers of tentacles average 25 in the largest polyps in each set. The male gonophore is like that of *T. spectabilis.* The female gonophore bears four tentacles, resembling *T. spectabilis* but the tentacles are not clumped.

Tubularia larynx and *Tubularia spectabilis* may occur mixed in collections and in the same bunches taken from the Canal during July.

Preliminary observations on maturation of the female gonangium of Campanularia flexuosa. RICHARD L. MILLER.

The mature female gonangium of *Campanularia flexuosa* secretes a sperm attractant from cells located at the rim of the aperture at the distal end of the gonangium. These cells arrive at this location as the result of a series of tissue movements which take place as the gonangium undergoes maturation. In the immature state the gonangium is composed of a blastostyle in the shape of a thin tube with a swollen distal end in the shape of a squat cylinder. The ectoderm cells in the cylinder secrete the perisarc which encloses the entire structure so that the blastostyle stalk is held centrally in a hollow perisarc tube. The egg-bearing sporosacs are arranged along the blastostyle stalk. The immature state involves growth in length with new sporosacs being added at the base of the stalk for about 3–5 days, followed by an abrupt (6–10 hour) metamorphosis of the distal blastostyle cylinder into the hollow funnel-like morphology of the mature gonangium.

The first sign of maturation is the appearance of a groove in the disto-lateral ectoderm of the blastostyle cap. The groove deepens and widens as the blastostyle cap within it pulls cleanly away from the overlying perisarc and shrinks proximally. The ectoderm outside of the groove spreads up the inside surface of the perisarc tube to its distal end and also down toward the eggs. The result of these movements is a funnel-shaped layer of flattened ectoderm cells which adheres to the inner edge of the aperture rim and taper down to the shrunken blastostyle cap.

Small spots of Nile Blue Sulfate dye were applied to selected areas on the distal and lateral surfaces of the blastostyle cap of immature gonangia. Spots placed above the groove moved with the blastostyle ectoderm and showed little spreading. Spots below the groove (on the sides of the cap) spread markedly, extending from the level of the upper sporasacs to the aperture rim. There appears to be a direct relationship between the amount of spreading and the distance from the center of the distal surface of the cap to the area where the dye is applied.

Supported by a grant from the American Cancer Society.

Fluorescence and time distribution of photon emission of bioluminescent photocytes in Obelia geniculata. James G. Morin and George T. Reynolds.

Biochemical studies of the bioluminescent system of *Obelia* provided evidence for a fluorescing chromophore attached to the luminescent photophore (photoprotein) in such a way that the energy from the photophore (λ_{max} 475 nm [blue]) was efficiently transferred to this chromophore which then emitted the light as a fluorescence at a longer wavelength (λ_{max} 505 nm [green]). This molecular arrangement has been exploited in order to observe the fluorescent sites *in vivo* by exciting the chromophore with 460 to 470 nm transmitted light using interference filters and then cutting off all light below 500 nm but allowing the fluorescent 505 nm light to pass by using the appropriate barrier filters. The sites that are made visible by this technique are green, discrete cells which frequently possess long cytoplasmic projections. They lie in the gastrodermis. These green fluorescing cells (photocytes) have also been shown to be the luminescent sites by simultaneous application of fluorescence microscopy and image intensification. Conversely, all luminescent sites showed green fluorescence.

Last year we reported here some of the temporal and spatial characteristics of these photocytes using photometric techniques. Image intensification has verified these results by incorporation of moving film recordings of the intensified image. Records have been made at 1, 2.5, 5, 10, 25, 50, and 100 cm/sec. They showed that individual photocytes flash repetitively in response to individual electrical stimuli in a train of such stimuli, each flash has a duration of about 65 to 75 msec with a rapid onset to maximum intensity and a relatively slow decay, a gradual increase in the interflash interval in a burst from about 60 msec to about 150 msec, a facilitation and decline of flash intensities within each burst, nonpolar spread of the luminescence, and a flash synchrony between adjacent photocytes.

Spectral sensitivity of single photoreceptors in the lateral and ventral eyes of Limulus. John Nolte and Joel E. Brown.

Spectral sensitivity curves for lateral eye retinular cells and ventral eye photoreceptor cells were determined from intracellular recordings of receptor potentials. The relative number of photons needed to elicit a response of arbitrary fixed amplitude was found at wavelengths from 325 nm to 650 nm, in steps of 25 nm. The sensitivity is defined as the reciprocal of this number. The spectral sensitivity curves all peaked at about 520 nm, but the curves obtained from normal lateral eyes with the stimulus delivered through the side of the ommatidium (*i.e.*, through the screening pigment) indicated relatively more sensitivity on the long wavelength side of 520 nm than did the curves obtained when the stimulus was delivered through the lens. The difference was about 1.5 log units at 650 nm. In the UV, the cells were also relatively more sensitive when light was presented through the side of the ommatidium; this is a consequence of an increase in optical density of the lens to light of short wavelength. In the long wavelength region, the curves obtained by delivering the stimulus through the lens are nearly identical to those obtained both from ventral eye photoreceptors, which have neither lens nor screening pigment, and from lateral eye retinular cells of a "white-eyed" mutant, when the light is delivered through the side of the ommatidium (which lacks screening pigment). Furthermore, the curves obtained from ventral eye photoreceptors are identical to those obtained from lateral eye retinular cells of white-eyed mutants throughout the spectrum. We conclude first, that the lateral eye screening pigment of *Limulus* is not black but rather red-transmitting and second, that lateral eye retinular cells and ventral eye photoreceptors contain the same photosensitive pigment.

Supported by NINDB Grants 5R01 NB07501–02 and 2R01 NB06251–04.

Cellular distribution of luminescent flashes in Gonyaulax, *mechanically stimulated by a piezoelectric suction pipette.* GEO T. REYNOLDS, J. W. HASTINGS AND R. S. NAGEL.

Studies of the location, distribution, and magnitude of light emission in various dinoflagellates are of interest in that they provide insight into the mechanism whereby flashing is controlled and into the structural organization of the light emitting system.

Stimulation of light emission at a precisely defined time is often difficult. Electrical and chemical methods have been used in the past in connection with image intensifier systems to study the bioluminescence of *Noctiluca, Pyrocystis* and *Gonyaulax*. However, recognition of the fact that in the natural environment flashing occurs in response to mechanical stimuli has led to the development of a new stimulating technique, which has been applied to a number of bioluminescent organisms. A thin walled ceramic cylinder with piezoelectric properties is incorporated as an integral part of a suction micropipette. This pipette is then used to hold the specimen in the field of view of the microscope in the conventional way. An electrical pulse, serving as a precise time reference for the remainder of the system, causes a shock wave to be transmitted down the pipette to the organism. In this way image intensifier studies of *Gonyaulax, Pyrocystis* and *Noctiluca* have been performed with ease.

The spatial distribution of light sources in the *Gonyaulax* flash have been found to be distinctly different from those in the other two dinoflagellates. The light appears to be distributed over a large fraction of the structure but not uniformly throughout the entire volume. The observations are consistent with light sources distributed in the outer 10–30% of the radius, and could be attributed to a distribution of these sources throughout the extranuclear cytoplasm. In some records a dark region resembling the nucelus appears as a shadow, suggesting that no light originates in this portion of the cell.

Reversal potential and conductance changes during the EPSP of eel electroplaques. (Electrophorus electricus). F. RUIZ-MANRESA.

The I-V relationship for the excitable membrane of single isolated electroplaques was obtained by current clamp techniques. The conductances and batteries for the steady state (K), peak of the spike (Na) and peak of the EPSP could be determined from the graphs. Advantage was taken of the linear steady state characteristic of this cell in the presence of $BaCl_2$ (2.5 mM) for more accurate extrapolations. The I-V relation of the EPSP was reproduced in each cell by carbachol (50–100 μg/ml). No differences were found in Cl free (Propionate) solution. Addition of carbachol in Na free (Tris chloride) solution, did not produce the depolarization that results from synaptic activation. Thus the depolarizing electrogenesis of the EPSP is due to the emf of a Na battery. Cl does not participate in the electrogenesis. The conductance changes during the EPSP appear to be only for Na and K. Typical values for E_K, E_{Na} and E_{EPSP} were −81, +136 and +30 mV, respectively. During synaptic activation the ratio G_{Na}/G_K calculated from the measurements was close to unity, but for different cells the individual conductances for Na and K ranged between 0.05 and 0.27 mho/cm^2.

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The author expresses his gratitude to Dr. H. Grundfest.

Interactions among the column contraction and tentacle contraction pacemakers in Hydra. NORMAN B. RUSHFORTH.

Electrical recordings from the base of hydra (*H. pirardi, H. pseudoligactis, H. fusca, H. littoralis,* $n = 7$) were employed to study the joint firing patterns of two pacemaker systems: (a) the column contraction system which produces CP's, and (b) the tentacle contraction system which produces TCP's.

The probability distributions of TCP's for a 30 second period before and after CP firing showed: (1) an increasing frequency of TCP firing, reaching a maximum 4–6 seconds before CP activity; (2) fewer TCP's within the 2 second period before a single CP or the first CP in a burst; (3) marked suppression of TCP's for 10 to 20 seconds after a CP burst, followed by gradual restoration of TCP activity; (4) lowered TCP frequency for 4–6 seconds after a CP single, followed by roughly 10 seconds of TCP suppression and then a gradual increase of TCP activity.

In CP bursts containing TCP's during inter-CP intervals, TCP's are most frequent in the first inter-CP interval and are virtually absent in subsequent intervals. An inter-TCP interval is greater when a CP burst is present than when a single CP occurs in the interval. Both of these intervals are significantly longer than intervals containing no CP's ($P < 0.001$).

These observations suggest loose coupling of the TCP and CP systems, resulting in frequent TCP's before CP firing, and inhibition of TCP's by activity of the CP system. It is postulated that TCP's weakly inhibit the CP system and CP's are associated with the decrease in TCP frequency. However, in some preparations during a vigorous CP burst the tentacles contract synchronously with contractions of the column. The post-TCP burst excitability of the CP system may result from processes similar to post inhibitory rebound. The TCP system, however, plays a minor role in the temporal patterning of CP's whereas CP's markedly suppress TCP activity.

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Prefertilization stimulation of recovery from radiation-induced mitotic delay in sea urchin eggs. RONALD C. RUSTAD, ARTHUR M. ZIMMERMAN AND MICHAEL A. GREENBERG.

Two different approaches were used to study repair of the radiation damage that leads to delay in cell division.

In one series of experiments, eggs of the sea urchin *Arbacia punctulata* were γ-irradiated. Some cells were then fertilized while others were incubated at 20° C for 10 minutes either at atmospheric pressure or at 7500 psi. After fertilization, the cells left for 10 minutes at atmospheric pressure were less delayed by irradiation than eggs which had been fertilized earlier. The pressure-treated cells divided much sooner than eggs recovering at atmospheric pressure. Pressurization of the unfertilized eggs also enhanced recovery from the γ-ray-induced damage to sperm that leads to mitotic delay. However, combination of radiation and pressure led to decreased survival at gastrula and pluteus stages.

In another series of experiments, unfertilized eggs were treated after the method of Kojima, with $1.7 \times 10^{-4}\,M$ neutral red or $8.6 \times 10^{-4}\,M$ sodium lauryl sulfate (SLS) to shorten the first division cycle without directly stimulating the cortical reaction or parthenogenesis. These treatments led to reduction of the radiation-induced mitotic delay caused by damaged sperm. In some experiments, the SLS-treated eggs fertilized with irradiated sperm divided even earlier than the control cells.

Thus, treatment of eggs with either high hydrostatic pressure or potential-parthenogenetic activating agents leads to reduction of the radiation-induced mitotic delay. The effects might be due to either initiation of events which permit the cell to bypass the mitotic stages normally delayed by radiation or to stimulation of repair mechanisms. If repair of radiation-induced lesions is involved, then it must occur after fertilization in the experiments with irradiated sperm.

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The cell cycle of the sea urchin Arbacia punctulata *as determined by ^{3}H-thymidine uptake and autoradiography.* SISTER ARLINE C. SCHMEER, O.P., MUSETTA HANSON, AND E. MICHAEL TANN.

The cytokinetics of the sea urchin, *Arbacia punctulata,* were calculated after the administration of a single pulse of tritiated thymidine and the utilization of autoradiographic methods. Since the early embryogenesis of the sea urchin is an excellent example of a naturally synchronous cell population, it was essential, for this study, to obtain representative stages of developing embryos (zygote through gastrula). To prepare an asynchronous population, fertilized eggs were introduced periodically into filtered sea water maintained at 19° C. Simultaneously, with the addition of the last zygotes, 250 μC of ^{3}H-TdR (New England Nuclear, specific activity 6.7 c/mM) was added to approximately 150 ml of the filtered sea water at zero time. The cells were exposed to a single 20 min pulse of the precursor, washed several times, sampled at specific intervals, fixed, and prepared for light microscopy and autoradiography. Optimal exposure of the cells under emulsion was 24 to 48 hr. Quastler and Sherman's techniques were used to determine the cell cycle from a curve describing the rate of appearance and disappearance of labeled mitoses. For each sampling interval a minimum of 250 mitotic figures was scored. The DNA synthetic index (DSI) was calculated by scoring the percentage of labeled cells per 1000 cells. The mitotic index (MI) was determined from the number of mitotic figures for every 1000 cells. A best line curve appeared as follows. At 35 min from time zero approximately 51% of the mitoses were labeled. The greatest percentage of labeled mitoses was 95%, scored at 40 min. This constituted the first ascending curve. A sharp decline followed at 45 min with 53% labeling until a second ascending curve was observed at 120 min with 61% of the mitoses labeled. A second plateau was reached at 150 min with 74% labeling, falling thereafter to 62% and essentially remaining at this level for all successive sampling intervals. From this cell cycle curve it was determined that $S = 10$ to 12 min; the maximum $G_2 + \text{prophase} = 35$ min; $M =$ 30 to 60 min; $G_1 = 0$, and the total generation time is approximately 105 min. The mean DSI was 51.67% $\pm$ 3.90 and the MI 19.89 $\pm$ 2.66.

This research was partially supported by the Father Arsenius Boyer Fellowship of the Marine Biological Laboratory, Woods Hole, Massachusetts; the Tad Beck Fund of Lincoln, Nebraska; The Cancer Research Fund, Ohio Dominican College, Columbus, Ohio; and the Ashland County Health and Ashland County Cancer Foundations, Ashland, Ohio.

The role of "contractile ring" filaments in dividing Arbacia *egg.* THOMAS E. SCHROEDER.

Cytokinesis of first cleavage in *Arbacia* (sea urchin) eggs is by equatorial constriction. Precise meridional sections at mid-cleavage, when examined by electron microscopy, reveal a dense exclusion zone (0.1 μ thick and 10 μ wide) immediately beneath the plasmalemma of the concave furrow. It appears as dense 50–100 Å dots centered about 200 Å apart. In nonfurrow regions at similar stages, and at equatorial surfaces during metaphase, no such dense zone occurs.

Precise equatorial sections through the constricted "necks" of half-cleaved eggs exhibit the dense zone as a continuous ring around the convex perimeter of the cell. The zone then appears exclusively composed of highly aligned, circumferentially oriented cytoplasmic filaments which measure 50–80 Å in thickness and are indefinitely long. It is inferred that dot-like profiles in meridional sections represent end-on views of sectioned filaments.

Within seconds after exposure to Cytochalasin B ($2 \times 10^{-7} M$), shallow cleavage furrows regress, and deep furrows progress no further. Mitosis, however, is unaffected. Cytochalasin B alters fine structure within a minute by causing complete disappearance of furrow filaments, retraction of most surface projections, and some blebbing.

Furrowing may be induced in unfertilized eggs by stratification and brief osmotic shock. After three hours, furrows appear, but they are not underlain by dense cytoplasm or filaments. Germinal vesicles and annulate lamellae remain intact.

Conclusions: The "contractile ring," postulated years ago as the organelle responsible for cytokinesis in *Arbacia*, is composed of fine filaments aligned circumferentially at the equator. Active constriction correlates with the presence of these filaments, whose mechanical action may be likened to a "purse string." Breakdown of the nucleus and/or annulate lamellae is necessary for the appearance of "contractile ring" filaments in *Arbacia*. The mechanism of furrowing as induced in unfertilized eggs is probably unrelated to the mechanism of normal cytokinesis.

Further studies of the effect of actinomycin-D on distal-hydranth regeneration in Tubularia: *chronological drug administration.* IRWIN I. SINGER AND JOHN D. PALMER.

During the regeneration of a new hydranth from the cut end of a *Tubularia* stolon, seven well defined morphological stages can be distinguished. We have previously demonstrated that subjecting a hydranth-amputated stolon to a 4-hour pulse of actinomycin-D (an inhibitor of DNA dependent RNA synthesis) augments the time needed to complete regeneration—the duration depending on the concentration of actinomycin used. For example, a concentration of 5.0 μg/ml, increases the regeneration time from 3 days to over 6 days.

The study reported here was designed to determine the time interval in the regenerative process during which maximum RNA synthesis takes place. Eight millimeter hydrocaulus segments—removed 3 mm below the hydranth—were subjected to 5.0 μg/ml actinomycin-D in millipore-filtered sea water for 4-hour periods, at hour 0, 4, 6, 12, 17, 22, 32, 36, and 42, after the moment of hydranth amputation. Care was taken to be sure that the proximal end of each segment was open just before immersion in actinomycin to allow the drug to enter the coelenteron. This concentration of the drug does not affect healing and the stolon ends close 2 hours after cutting. The segments were washed after the drug treatment, but the actinomycin was not removed from the coelenteron. At least 20 stolon segments were used for each treatment, and the progress of distal-hydranth regeneration was observed at 24-hour intervals for 6 days for each of the chronological groups, and for untreated controls. The regenerates were maintained at 14° C throughout the experiment.

Regeneration is stolons treated at hours 0, 4, 22, and beyond was delayed, but was usually completed by day 6. Those whose drug treatment began at 6, 12, and 17 hours were not completely regenerated by this time. Therefore, it is concluded that RNA synthesis associated with hydranth regeneration takes place mainly between hours 6 and 22 after amputation.

A fine structural study of lipid-uptake in the terminal intestine of the scup, Stenotomus chrysops. ELLIOTT W. STRAUSS AND SUSUMU ITO.

This study was undertaken when it was noted that proximal and distal intestine of the scup possessed different capabilities for absorption of luminal materials. Fish were tube-fed orally with 0.5 ml of corn oil (Mazola) per 100 g body weight. Others were given ferritin, 50 mg per 100 g fish, or a mixture of ferritin and corn oil. Controls received sea water. Animals were sacrificed at 1–24 hours and samples fixed for electron microscopy. The absorptive cells in upper intestine generally resembled intestinal cells of mammals. Morphological features of lipid absorption also were similar. However, absorptive cells in the terminal intestine, 1–2 cm proximal to the cloaca, were strikingly different from the proximal cells. Apical cytoplasm of distal cells contained abundant tubular, vesicular, vacuolar and lysosomal structures, the former penetrating completely through the terminal web region. After feeding corn oil alone or with ferritin, distal cells contained cytoplasmic lipid droplets and increased numbers of enlarged lysosomal bodies, but lipid was not seen entering the cells. The tubulo-vesicular system of membranes accumulated ferritin in one hour. By 12 hours after feeding ferritin alone, lysosomal bodies were packed densely with the protein granules in a crystal-like array. When a mixture of lipid and ferritin was given, an admixture of lipid and ferritin could not be identified certainly in the same organelle. Nor could the uptake of lipid be seen. These observations are interpreted to indicate molecular or micellar fat was taken up in the upper intestine of scup. However, in the distal intestine, the intensive pinocytosis suggests a mechanism for taking up the lipid in bulk.

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Protein synthesis during hydranth regeneration in Tubularia. KARL S. WITTMAN.

The cut stem of *Tubularia* regenerates its hydranth in about 3 days at 16°–17° C. The objectives of this investigation were (1) to determine if any changes in the incorporation of labeled amino acid into TCA-insoluble material occurs during cellular reorganization of the cut stem and differentiation of a new hydranth, and (2) if inhibition of protein synthesis affects hydranth development.

Hydranths were removed and 5–7 mm pieces of the stem were placed in stender dishes kept at 16°–17° C. Controls were incubated in filtered sea water containing C^{14}-leucine (0.1 μc/ml; > 250 mc/mM leucine specific activity) and penicillin (250 units/ml). Experimentals were similarly incubated but with the addition of puromycin at a concentration of 20 μg/ml. At intervals during the first 75 hours, samples from each group were removed and TCA-insoluble material assayed using a Nuclear-Chicago surface counter.

During the first 20 hours no increase in incorporation of label occurred in control or puromycin-treated stems and the level of incorporation was similar in both groups. From 25 to 75 hours there was a notably higher increase in incorporation of label in controls.

Puromycin-treated stems required a longer time to regenerate and only 90% of the specimens formed hydranths. These were smaller and had shorter proximal and distal tentacles than the controls.

When puromycin was removed at 25 hours (prior to the pigmentation stage) and the stems allowed to continue regeneration in fresh sea water, 100% of the stems formed normal hydranths. When administration of puromycin was delayed until 25 hours, 85% of the stems formed hydranths which were all small and had short tentacles.

This work suggests that (1) depression of protein synthesis markedly inhibits the rate of regeneration and affects the quality of hydranth formation, (2) the puromycin sensitive period occurs during overt regeneration, and (3) protein synthesis may not be essential during cellular reorganization of the first 25 hours.

This study was supported by a Faculty Fellowship and Grant-in-Aid from the State University of New York Research Foundation.

UV and visible light effects on dogfish eye tissues. S. ZIGMAN AND S. J. BAGLEY.

The effects of 365 mμ UV light on protein synthesis in dogfish (*Mustelus canis*) retinal rods, and the mechanism whereby incandescent light causes constriction of the pupil after removal of the posterior half of the eye were studied.

Rods were isolated from whole retinas (dissected in dim red light) by shaking in ice-cold elasmobranch Ringer's solution (containing 0.3 M urea) after incubation in urea-free Ringer's (isotonicity adjusted with $NaHCO_3$). By scanning electron microscopy, these rods were shown to be intact inner and outer segments (length 60 μ, width 10 μ). Rods of 6.5×10^{-6} mg dry weight contained 1.7×10^{-3} μg protein, 0.25×10^{-3} μg RNA, and 0.33×10^{-3} μg DNA each.

When rods were illuminated with 500–600 ft-c of cool-white fluorescent light, the incorporation of C^{14}-amino acids into protein was depressed by 70% (isolated rods incubated 12 hr plus 10 μc of C^{14} per 5 ml). When 5000 $\mu W/cm^2$ of 365 mμ UV light were employed, an inhibition of 70% of C^{14}-amino acid incorporation into rod protein was observed during 8 hr of incubation (10 μc of C^{14} per 5 ml). The 365 mμ portion of fluorescent lamps apparently leads to diminution of protein synthesis in rods and other cells. Vitamin A, a strong absorber of UV, is important in cell growth and tissue maintenance and seems to contain the chromophore. The presence of retinene 1 (maximum absorption at 365 mμ) would render rods more susceptible to damage than other cells. Conversion of absorbed light to heat may also be inhibitory. Only 90% of 365 mμ light is absorbed in the eye before reaching the rods (lens absorptions is 40–50%).

When anterior halves of eyes were illuminated by room light (50–100 ft-c), or by more intense light (300 ft-c) the iris contracted maximally in 1 minute (at 8° C). When returned to the dark for several minutes, pupillary dilation recurred. This process continued for several days if the anterior segment was maintained in cold Ringer's solutions in the dark. Contraction occurred twice as fast at room temperature as at 8° C, was not effected by 2% atropine, but ceased in $10^{-3}\,M$ CN. These results confirm previous work proving that no nervous control over iris sphincter muscle contraction exists in this purely photosensitive reaction.

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Experimental furrow induction in activated Arbacia *eggs.* ARTHUR M. ZIMMERMAN AND SHUHEI YUYAMA.

Although furrowing in sea urchin eggs does not usually occur until mitotic events are complete, it has previously been demonstrated that furrowing could be induced in fertilized *Arbacia* eggs by pressure-centrifugation treatment. The current work represents an attempt (1) to investigate furrow induction in parthenogenetic eggs, and (2) to determine the thiol composition of the KCl soluble protein, following pressure-centrifugation, and (3) to measure the gelational state of the cytoplasmic cortex of parthenogenetic eggs.

Furrow induction was successfully initiated in butyric acid-hypertonic sea water activated eggs of *Arbacia punctulata* following pressure-centrifugation treatment. Whereas the frequency of induction in fertilized eggs is greatest following 4 minutes of pressure-centrifugation (12,000 psi at 33,000 g), artificially activated eggs show maximal induction after only 2.5 minutes. In many experiments, parthenogenetic eggs showed virtually 90–95% furrow induction 2–5 minutes after treatment. In contrast to the foregoing result, the nonpressurized controls were markedly asynchronous and often only 10–20% furrowed after 2 hours. In certain experiments it was possible to demonstrate furrow induction in anucleate fragments which were activated with sperm (fertilized merogones), however, it was not possible to induce furrowing in parthenogenetic merogones.

Preliminary studies on the total sulfhydryl composition of the $0.6\,M$ KCl soluble protein indicate that there is a 8–12% increase in the total SH groups after pressure-centrifugation treatment. These results support the concept that there is a change in the SH composition of the cortical protein prior to furrowing.

Pressure-centrifuge measurements of the structural state of the cortical cytoplasm of parthenogenetic eggs were made at various pressures (6000–14,000 psi) at 22.8° C employing a centrifugal force of 33,000 g. The artificially activated eggs yielded a value for the cortical gel which was 20% lower than that found in the controls.

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INDIVIDUAL AND COMBINED EFFECTS OF SALINITY AND TEMPERATURE ON EMBRYOS AND LARVAE OF THE COOT CLAM, *MULINIA LATERALIS* (SAY)[1]

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Thorson's 1946 publication on the early life history of Danish marine bottom invertebrates has helped establish the field of marine benthic and larval ecology. A notable lack of knowledge still exists, nevertheless, regarding the life history and autecology of the majority of organisms that are part of bottom communities.

Until recently the rearing of marine bivalves was virtually impossible because of the lack of reliable methods. The relatively new development of techniques for rearing marine bivalves (Loosanoff and Davis, 1963) has provided further incentive, however, for autecological studies of larvae. By the successful conditioning and spawning of adult bivalves the larvae of many species have been reared under controlled conditions (Loosanoff and Davis, 1963; Stickney, 1964; Walne, 1964, 1966; Bayne, 1965; Chanley, 1965a, 1965b, 1966, 1967; Chanley and Castagna, 1966; Loosanoff, Davis and Chanley, 1966). These techniques make it possible to subject bivalve embryos and larvae to various ecological factors under controlled experimental conditions.

Little is known about the combined effect of two or more environmental factors on marine animals. Medcof and Needler (1941) attempted to deduce the interaction of temperature and salinity on the condition index of the American oyster, *Crassostrea virginica*, in natural waters. Costlow, Bookhout, and Monroe (1960, 1962) studied the combined effects of temperature and salinity on development of eggs and larvae of the decapod crustaceans, *Sesarma cinereum* and *Panopeus herbstii*. Kinne (1963, p. 302) reviewed the existing knowledge of the effects of temperature and salinity on marine and brackish water fish and emphasized the fact that "monofactorial analysis may lead to conclusions that are ecologically invalid," and "should be replaced wherever possible by bi, tri, or polyfactorial approach." This view influenced Davis and Calabrese (1964) to study the effects of variations in such factors as food and salinity on the temperature

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tolerance of embryos and larvae of the American oyster, *C. virginica,* and the hard shell clam, *Mercenaria mercenaria.*

The present study of the individual and combined effects of salinity and temperature on embryos and larvae of *Mulinia lateralis* (Say) shows the interaction of these factors in the laboratory; the tolerances established, however, may be further modified in nature where other environmental factors may also affect the temperature and salinity tolerances of these clams.

METHODS

Methods for maintaining bivalves in spawning condition and obtaining fertilized eggs throughout the year were described by Loosanoff and Davis (1963). To determine the effect of salinity and temperature, individually and in combination, on embryonic development of *M. lateralis,* 12,000 to 15,000 fertilized eggs, from parents that were held at the same initial temperature and salinity, were placed into each of a series of 1-liter, polypropylene beakers. To determine the effect of the different factors on embryonic development, the larvae from each culture were collected, after 48 hours at the experimental conditions, on a stainless steel screen of mesh size small enough to retain them. The larvae were resuspended in a 250-ml graduated cylinder and, after thorough stirring to insure uniform distribution of the larvae, a 4-ml quantitative sample was removed and preserved in 5% neutral formalin. The samples were examined under a compound microscope (× 125) and the number of larvae that had developed normally was counted. To compare the number of embryos developing to normal straight-hinge larvae in successive experiments, the results were calculated as the percentage of the maximum number developing normally at any salinity and temperature (either individually or in combination) in that experiment.

To ascertain the effect of the various test conditions on survival and growth, I placed 10,000 to 14,000 larvae, which had been reared to the 48-hour straight-hinge stage under normal conditions (27 ± 0.5‰ salinity and 25 ± 1° C), into each of the series of 1-liter beakers. The sea water in all cultures was changed 3 times a week to eliminate metabolic waste products, and experimental conditions were re-established. In all experiments 50 mg/l of Sulmet were routinely added to all cultures to prevent possible disease-induced mortality that was not a direct effect of the factor being tested. (Sulmet, sodium sulfamethazine, is a trade name of American Cyanamid Co. Mention of trade names does not imply endorsement of the product by the Bureau of Commercial Fisheries.) In experiments involving only temperature, supplemental algal food, consisting of a mixture of *Isochrysis galbana, Monochrysis lutheri,* and *Chlorella* sp. 580 (Indiana University Collection #580), was added daily by the procedures of Davis and Guillard (1958). To keep salinities constant in tests involving salinity, however, food was added only when the water was changed and salinities were adjusted. Experiments were discontinued when larvae in the fastest growing cultures reached setting size (6 to 8 days). When the experiments were terminated, quantitative samples were taken as in the experiments on embryonic development. The number of larvae that had survived the experimental treatment was counted and 100 (if available) were measured to the nearest 5 μ with an ocular micrometer in a compound microscope. The number of larvae that survived and the increase in mean length in each experiment were calculated as percentages of the maximum number that survived and the increase in length of larvae in the most rapidly growing cultures, respectively, at any salinity and temperature (either individually or in combination) in that experiment. The method for determining the number of larvae surviving or the percentage of bivalve embryos developing into normal straight-hinge larvae is considered accurate to about ± 10% (Davis, 1958).

Four experiments were conducted at salinities of 7.5 to 37.5‰ at 2.5‰ intervals. The effect of each salinity was tested in at least 2 experiments and some salinities were repeated in all 4 experiments. All cultures were kept in a constant temperature bath at 25 ± 1° C. Salinities below 27‰ were prepared by diluting laboratory sea water with demineralized tap water, and salinities above 27‰ were made up by adding sea water, concentrated by evaporation, to the laboratory sea water. All salinities were determined by the hydrometer method and the use of Knudsen's Tables (1901). Eight experiments were conducted at temperatures of 7.5 to 34.5° C at 2.5° C intervals. The effect of each temperature was tested in at least 2 experiments and some temperatures were repeated in as many as 5 experiments. Duplicate cultures were established at each test condition. The salinity of the sea water in these experiments was 27 ± 0.5‰.

Three experiments were conducted with salinities of 10 to 35‰ at 5‰ intervals in combination with temperatures from 7.5 to 32.5° C at 5° C intervals. Duplicate cultures were established at each of the 6 salinities and 6 temperatures tested, giving a total of 36 combinations and 72 cultures in each experiment. Low salinity sea water was made up by dilution with demineralized tap water. I obtained highly concentrated sea water by first freezing some laboratory sea water and then later melting off just a small portion. This highly concentrated sea water was then used to make up the sea water for high salinity studies.

TABLE I

Percentage of embryos developing, larvae surviving, and increase in mean length of larvae of M. lateralis *at different salinities*

Salinity* (o/oo)	Percentage development of embryos	Percentage survival of larvae	Percentage increase in mean length of larvae
7.5	0.0	5.8	6.0
10.0	0.0	19.1	19.4
12.5	0.0	35.0	36.7
15.0	10.1	32.6	42.4
17.5	15.6	58.6	61.9
20.0	59.8	87.7	78.7
22.5	83.5	83.4	78.9
25.0	91.4	93.7	100.0
27.5	97.0	94.6	82.4
30.0	81.1	61.4	75.1
32.5	61.9	64.4	68.8
35.0	14.9	38.5	54.0
37.5	1.2	40.7	61.7

* All cultures were maintained at 25 ± 1° C.

Results

Effect of salinity

M. lateralis embryos developed into normal straight-hinge larvae throughout the relatively wide salinity range from 15 to 35‰ at 25 ± 1° C (Table I and Fig. 1). At a salinity of 37.5‰ only 1.2% of the embryos developed into normal shelled

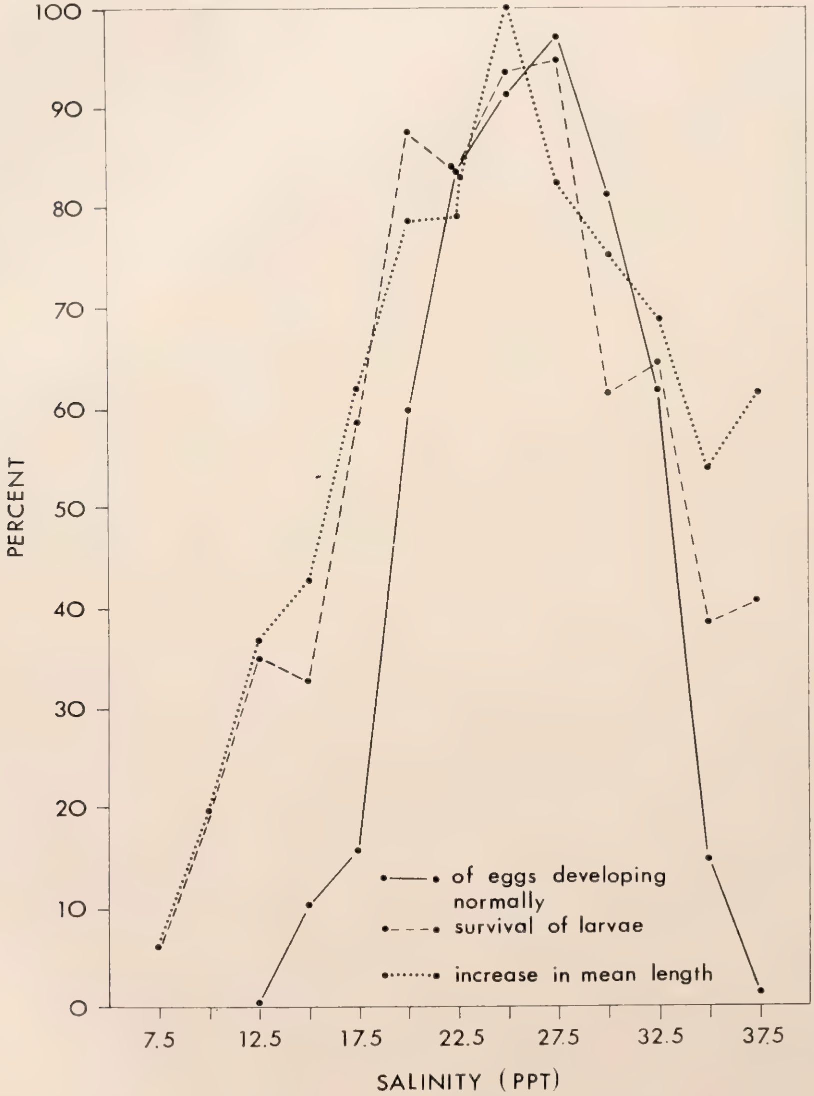

Figure 1. The salinity tolerance of embryos and larvae of *M. lateralis* at 25 ± 1° C, as indicated by percentage of embryos that developed normally, percentage of larvae that survived, and percentage increase in mean length of larvae.

TABLE II

Percentage of embryos developing, larvae surviving, and increase in mean length of larvae of M. lateralis *at different temperatures*

Temperature* (°C)	Percentage development of embryos	Percentage survival of larvae	Percentage increase in mean length of larvae
7.5	0.0	86.1	3.7
10.0	17.3	81.0	5.7
12.5	60.9	89.7	14.2
15.0	75.1	92.7	25.3
17.5	92.8	85.8	41.9
20.0	98.9	93.5	69.5
22.5	92.4	92.0	75.6
25.0	92.1	90.4	79.2
27.5	43.3	88.4	93.5
30.0	39.0	60.1	79.4
32.5	0.0	29.9	64.2
34.5	—	0.0	0.0

* All cultures were maintained at 27 ± 0.5‰ salinity.

larvae, and at 35‰ 14.9% developed normally. At 15 and 17.5‰ 10.1 and 15.6%, respectively, of the embryos developed normally, and at 12.5‰ none developed. Approximately 60% of the embryos developed into shelled larvae at 20 and 32.5‰, but the salinity range for a satisfactory percentage of fertilized eggs (70% or more of maximum) to develop was from 22.5 to 30‰. With temperature constant at 25 ± 1° C the optimum salinity for embryonic development, *i.e.*, the salinity at which the highest percentage of straight-hinge larvae was obtained, was 25 to 27.5‰. Thus, the salinity range from 22.5 to 30‰ for satisfactory development of embryos into normal shelled larvae approximates the salinity range of 22.5 to 30‰ for satisfactory development of hard shell clam embryos, as reported by Davis (1958).

Once larvae reached the straight-hinge stage, some were able to survive at salinities from 7.5 to 37.5‰ (Table I and Fig. 1), a range much wider than that tolerated by developing embryos. Only a small percentage (5.8) of larvae, however, survived at 7.5‰, and only 32.6% at 15‰. The upper salinity limit for larval survival was not determined since at 37.5‰, the highest salinity tested, a rather high percentage (40.7) of larvae survived. A satisfactory percentage (70% or more of maximum) survived at salinities from 20 to 27.5‰, and optimum survival was at 25 to 27.5‰. Thus, the optimum salinity for both developing embryos and survival of larvae was 25 to 27.5‰.

Larvae were able to grow satisfactorily (70% or more of maximum) within the salinity range from 20 to 30 or 32.5‰; the optimum growth was at 25‰ (Table I and Fig. 1). The range for satisfactory growth was, therefore, slightly greater than that for satisfactory survival. Growth was most limited at 7.5 and 10‰ (less than 20% of maximum), but the rate of growth increased gradually as the salinity was increased from 12.5‰ (36.7%) to 17.5‰ (61.9%). The rate of growth decreased slowly at salinities above 27.5‰ but even at 37.5‰ (the highest salinity tested) the increase in mean length of larvae (61.7%) was substantial.

Effect of temperature

At a constant salinity of 27 ± 0.5‰ *M. lateralis* embryos did not develop at 7.5° C, but at 10° C a small percentage (17.3) developed into straight-hinge larvae (Table II and Fig. 2). The percentage of fertilized eggs developing normally increased sharply from 17.3 at 10° C to 60.9 and 75.1 at 12.5 and 15° C, respectively. Differences in the percentage of straight-hinge larvae obtained at temperatures of 17.5, 20, 22.5, and 25° C were not significant, although the data indicate that 20° C may be near the optimum. The percentage of embryos that developed normally was greatly reduced at 27.5 and 30° C (43.3 and 39, respectively) and none developed at 32.5° C.

Although no embryos developed at 7.5° C, it was evident that larvae could tolerate 7.5° C at least for 10 days with little mortality (Table II and Fig. 2). A total of 86.1% of the larvae survived at 7.5° C, but growth was negligible. Even though the larvae were capable of surviving this cold temperature for the duration of the laboratory experiments, growth was so slow as to make it obvious that all would have eventually died before metamorphosis. More than 80% of the larvae survived at temperatures from 7.5 to 27.5° C, but the percentage dropped sharply to 60.1 at 30° C, and no larvae survived at 34.5° C.

The average rate of growth of larvae increased progressively with each increase in temperature from 7.5 to 27.5° C and then progressively decreased at temperatures of 30 and 32.5° C (Table II and Fig. 2). Growth was satisfactory only within the range from 20 to 30° C. Larvae grew most rapidly at 27.5° C, the temperature found optimum for growth of larvae of the European oyster, *Ostrea edulis* (Davis and Calabrese, 1969).

Combined effects of salinity and temperature

The temperature and salinity requirements for development of *M. lateralis* embryos to straight-hinge larvae are shown in Table III. From 83.3 to 95.9% of the embryos developed into straight-hinge larvae within the dash-lined boundary which was circumscribed by temperatures from 12.5 to 27.5° C and salinities from

TABLE III

Percentage development of M. lateralis *embryos to straight-hinge larvae at different combinations of salinity and temperature*

Salinity (o/oo)	Temperature (°C)					
	32.5	27.5*	22.5	17.5	12.5*	7.5
35.0	1.0	41.3	58.5	42.8	2.6	0.0
30.0	20.3	88.5	93.9	89.1	83.4	0.0
25.0	31.6	84.8	95.9	91.8	69.2	0.0
20.0	27.3	84.5	91.4	83.3	41.7	0.0
15.0	1.5	6.4	12.8	0.7	0.0	0.0
10.0	0.0	0.0	0.0	0.0	0.0	0.0

* Results of two experiments—temperature control unit went out of order in third experiment.

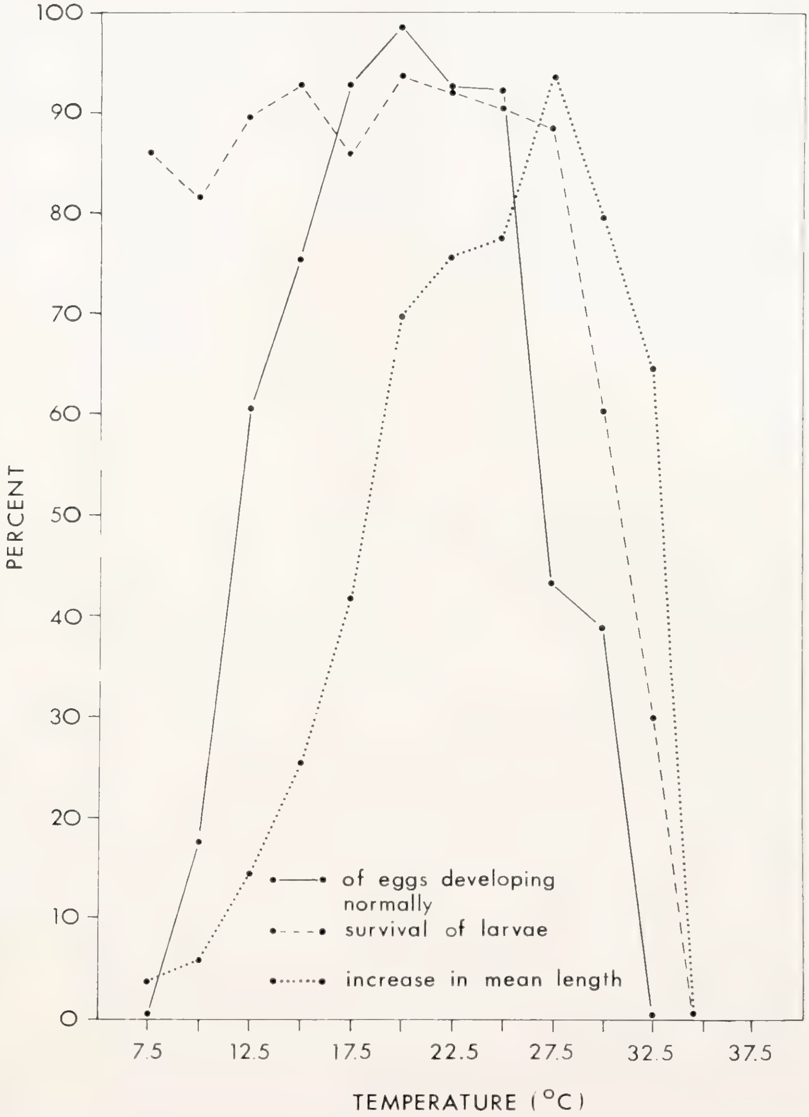

FIGURE 2. The temperature tolerance of embryos and larvae of *M. lateralis* at 27 ± 0.5‰ salinity, as indicated by the percentage of embryos that developed normally, percentage of larvae that survived, and percentage increase in mean length of larvae.

20 to 30‰. At 32.5° C the percentage of embryos that developed normally was drastically reduced at all salinities and at 7.5° C no normal larvae developed at any salinity. The temperature of 12.5° C appears to be "borderline" in that a normal number of embryos developed into straight-hinge larvae at 30‰ salinity, but 69.2% developed at 25‰ (this percentage is almost precisely at the 70% level

TABLE IV

Percentage survival of M. lateralis *larvae at different combinations of salinity and temperature*

Salinity (o/oo)	Temperature (°C)					
	32.5	27.5	22.5	17.5	12.5	7.5
35.0	32.1	76.2	83.4	92.6	84.7	75.1
30.0	75.1	82.4	92.6	89.8	84.3	75.2
25.0	32.2	77.9	90.8	88.2	81.6	80.7
20.0	58.7	82.7	82.9	85.8	83.1	82.3
15.0	25.4	75.9	85.6	79.3	80.7	75.9
10.0	2.2	63.9	77.7	77.9	66.5	62.4

previously discussed as of ecological significance), and 41.7% of the embryos developed normally at 20‰. At 35‰ salinity the percentage of embryos developing normally depended upon temperature, but at none of the temperatures tested did as many as 70% develop into normal straight-hinge larvae. A maximum of 58.5% developed normally at 22.5° C and 35‰ salinity. This salinity was obviously above optimum and approached the upper limit for embryonic development of *M. lateralis.* Only a few normal larvae were obtained at 15‰ salinity; the highest percentage (12.8) was at 22.5° C. No normal larvae developed at 10‰ at any temperature. For a satisfactory percentage of embryos to develop into straight-hinge larvae the salinity must remain between 20 and 30‰ and the temperature from 12.5 to 27.5° C (or 17.5° C if salinity is below 25‰).

Once the embryos developed to straight-hinge larvae, more than 70% survived at almost all combinations of temperature and salinity tested (Table IV). Erratic survival at 32.5° C, however, indicates that this temperature approached the upper limit for survival; only at a salinity of 30‰ was survival at this temperature above 70%. At low temperatures (12.5 and 7.5° C) survival of larvae was satisfactory at all salinities except 10‰, although growth was almost nil (Table V). At 10‰

TABLE V

Percentage increase in mean length of M. lateralis *larvae at different combinations of salinity and temperature*

Salinity (o/oo)	Temperature (°C)					
	32.5	27.5	22.5	17.5	12.5	7.5
35.0	63.5	83.6	70.2	25.4	2.7	1.2
30.0	65.0	99.4	90.5	43.1	3.2	0.7
25.0	45.6	81.0	82.3	43.2	4.9	0.7
20.0	61.7	84.6	92.9	61.7	13.3	1.1
15.0	38.8	57.5	68.2	38.6	3.5	1.2
10.0	0.0	40.9	44.9	15.8	0.9	0.5

salinity, even though survival was about normal at temperatures of 17.5 and 22.5° C and fair at 7.5, 12.5, and 27.5° C, growth was poor.

It is obvious from Tables III, IV, and V that larvae can survive throughout a wider range of temperature and salinity than is satisfactory for either embryonic development or growth of larvae. In these experiments growth of larvae was satisfactory only at temperatures from 22.5 to 27.5° C and salinities from 20 to 35‰. Temperatures of 17.5° C and lower were obviously below optimum, and 32.5° C was above optimum for growth. Growth, like survival, was erratic at 32.5° C. This temperature favors bacterial growth; probably the effects on survival and growth of larvae were the combined results of the increased bacteria and bacterial toxins and the reduced resistance of the larvae.

Satisfactory areas for reproduction of *M. lateralis* would appear to be limited to those where salinities are 20‰ or higher, as determined by the limits for embryonic development and growth, and where temperatures are approximately 22.5 to 27.5° C, as determined by the limits for satisfactory growth.

Discussion

Temperature has the greatest influence on the duration of the pelagic stage. Low water temperatures prolong pelagic life and high temperatures shorten it (provided food is adequate). Temperatures during the actual spawning season of *M. lateralis* in Long Island Sound never surpass the high limits that are lethal to developing eggs and larvae. Thorson (1950) suggested that the area of distribution of a species may comprise several sub areas in relation to temperature: one in which the temperature range permits the adult animals to grow, a somewhat more restricted area in which it permits development of gametes and spawning as well as growth (spawning appears to require a somewhat higher temperature than the process of ripening), and an even smaller area in which embryos and larvae successfully develop. Thorson also stated that temperatures required for spawning are so high and definitely limited that they will normally allow fertilized eggs and larvae to develop regularly, provided that other conditions are favorable; the same holds true for *M. lateralis*. Adults have survived temperatures at least as low as −2° C in outdoor tanks at the Milford laboratory and at the highest temperatures normally found in Long Island Sound (about 24° C). Gametogenic activity occurred at temperatures from about −0.1° C to normal spawning temperatures of about 20° C. From laboratory experiments it was determined that eggs developed from 10 to 30° C, and larvae survived at temperatures from 7.5 (the lowest tested) to 32.5° C.

Davis and Calabrese (1964) suggested that the failure of bivalve larvae to grow at low temperatures appeared to be caused by their inability to digest available food. Their experiments demonstrated that larvae of the American oyster and hard shell clam survived for long periods and ingested food at temperatures below the minimum at which they grew. They also suggested that enzymes required to digest naked flagellates, such as *I. galbana* and *M. lutheri,* were perhaps active at lower temperatures than those involved in the digestion of certain other forms with thick cell walls, such as *Chlorella*. Davis and Calabrese (1964) stated that the increase in growth rate of larvae at higher temperatures probably resulted from increased activity of the enzyme system at the higher temperatures. Ukeles (1961) demonstrated that temperatures of 27° C or higher destroyed the cells of *I. galbana* and *M. lutheri,* although cells of *Chlorella* sp. 580 survived even at 33° C. The reduction in growth of *M. lateralis* larvae at 32.5° C, then, may have represented partly an indirect effect of temperature on the food organisms. Since reduction of larval survival was also drastic at this temperature, it can be concluded that temperature also affected survival directly and, perhaps, growth. Davis and Calabrese (1969) studied the effect of temperature on European oyster larvae using the same 3 species of algal foods mentioned above. They believed that these foods were probably adequate at both temperature extremes, but did not preclude the possibility that other species of algae might have provided better growth at even lower or higher temperatures.

Salinity is a relatively stable factor in Long Island Sound; as with temperature, however, the area of distribution of an organism may comprise one in which the salinity range permits the adult animals to grow, possibly a smaller area in which the salinity permits development of gametes and spawning as well as growth, and an even more restricted area in which successful development of embryos and larvae is possible. Breuer (1957), in his studies of Alazan Bay, Texas, found *M. lateralis* in areas with salinities ranging from 1.4 to 75.1‰ and averaging 50.7‰. This extremely wide range does not necessarily mean that *M. lateralis* from all geographical areas are able to withstand such extreme salinities, nor does it mean that all stages of reproduction are accomplished at the extremes of the salinity range. In my studies I determined that embryos of *M. lateralis* from Long Island Sound did not develop below 15‰, and that at 37.5‰ (the highest salinity tested) only a negligible percentage developed. For an adult population to be established in areas of extreme salinity, one of two conditions would be necessary: (1) the salinity in that area would have to be within the range for embryonic development during the spawning season, or (2) spawning and embryonic development could take place in areas of suitable salinity and the larvae carried to areas of extreme salinity through dispersal. Additional research is needed to determine the minimum salinity at which *M. lateralis* develop gonads and whether the salinity at which the parent stock develops gonads influences the salinity tolerance of embryos and larvae.

I thank the following members of the Milford laboratory for their assistance: Dr. James E. Hanks and Mr. Harry C. Davis for many helpful suggestions throughout this study and for constructive criticism of this manuscript; Dr. Ravenna Ukeles for providing algal food for feeding *M. lateralis* larvae; Mr. Manton L. Botsford for preparation of the figures; and Miss Rita S. Riccio for her editorial review.

SUMMARY

1. Embryos of *M. lateralis* held at 25 ± 1° C developed satisfactorily (70% or more of maximum) within the salinity range from 22.5 to 30‰; 27.5‰ was optimum. Some embryos developed normally, however, at salinities as low as 15‰ (10%) and as high as 37.5‰ (1.2%).

2. Some larvae survived at all salinities tested (7.5 to 37.5‰); survival was 70% or more only within the range from 20 to 27.5‰.

3. Larvae grew satisfactorily within the salinity range from 20 to 30 or 32.5‰; 25‰ was optimum.

4. Embryos held at 27 ± 0.5‰ salinity developed satisfactorily within the temperature range from 15 to 25° C; 20° C was optimum. Some embryos developed normally, however, at temperatures as low as 10 (17.3%) and as high as 30° C (39%).

5. Some larvae survived at temperatures from 7.5 (lowest tested) to 32.5° C; survival was satisfactory from 7.5 to 27.5° C.

6. Larvae grew satisfactorily at temperatures from 20 to 30° C; 27.5° C was optimum.

7. The effects of salinity and temperature were significantly related only when the tolerance of either one or the other was approached. When the salinity was unfavorable, the range of temperature was markedly narrowed and, conversely, when both salinity and temperature were within the satisfactory range there was no significant interrelationship.

8. The range of temperature tolerance for embryos narrowed above and below 30‰ salinity, and salinity tolerance narrowed above and below 22.5° C.

9. Survival of larvae was relative uniform at temperatures of 7.5 to 27.5° C and at salinities from 10 to 35‰, but at 32.5° C (at all salinities other than 30‰) the percentage of larvae surviving was drastically reduced.

10. Growth of larvae was most rapid within the salinity range from 20 to 35‰ and within the temperature range from 22.5 to 27.5° C.

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THE DILATABLE RING CANALS OF THE OVARIAN CYSTOCYTES OF HABROBRACON JUGLANDIS

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The insects possessing polytrophic, meroistic ovarioles belong to the adephagous Coleoptera, the Dermaptera, Siphunculata, Neuroptera, Mecoptera, Lepidoptera, Diptera, and the Hymenoptera (Imms, 1957). In such insects the oocyte is a member of a cluster of sister cells. The other cells of the cluster function as nurse cells. Interconnections have been observed between the nurse cells and the oocyte in insects belonging to the Hymenoptera, Coleoptera, Lepidoptera, and Diptera (Brown and King, 1964; King and Aggarwal, 1965). In the fruit fly, *Drosophila melanogaster,* each of the egg chambers that reside in the vitellarium contains an oocyte and fifteen nurse cells. The oocyte and its interconnected nurse cells are fourth generation descendants of a single cell, a cystoblast, that resides in the germarium. The interconnected cells formed by the division of a cystoblast are called cystocytes (Brown and King, 1964). Sister cystocytes are connected by canals, each of which is surrounded by a ring-shaped rim. A stream of nurse cell cytoplasm rich in mitochondria, lipid droplets, and ribosomes passes through these ring canals and into the ooplasm (King, 1960; Cummings and King, 1969). It has been shown that the vast majority of ribosomes found in the mature *Drosophila* oocyte are derived from sister nurse cells (Koch, Smith and King, 1967; Dapples and King, 1970).

This paper presents the results of an ultrastructural study of the ring canals found in the germarial region of an ovariole from the parasitic braconid wasp, *Habrobracon juglandis* (Ashmead). This species is also referred to in the literature as *Microbracon hebetor* and *Bracon hebetor.* By studying serial transverse sections through several canals, it was possible to arrive at an understanding of the three-dimensional ultrastructure of the canal rim and to demonstrate that the rim is constructed in a manner that allows it to dilate.

MATERIALS AND METHODS

Wild type female wasps from Whiting stock 33 reared at 30° C upon larvae of the Mediterranean flour moth, *Ephestia* (= *Anagasta*) *kühniella,* provided the ovarian material. Ovarioles from newly emerged adults were fixed in a 0.2 *M* sodium cacodylate buffer solution (pH 7.45) containing 4% glutaraldehyde. The tissue was passed through six, thirty-minute changes of the glutaraldehyde fixative at 4° C. Next the tissue was washed by passing through five-, one-hour changes of glutaraldehyde-free buffer. After washing, the tissue was postfixed for two hours in cold 1% OsO_4 in the same buffer. Rapid dehydration in an ethanol series followed, and after a transfer to propylene oxide, the ovarioles were infiltrated with

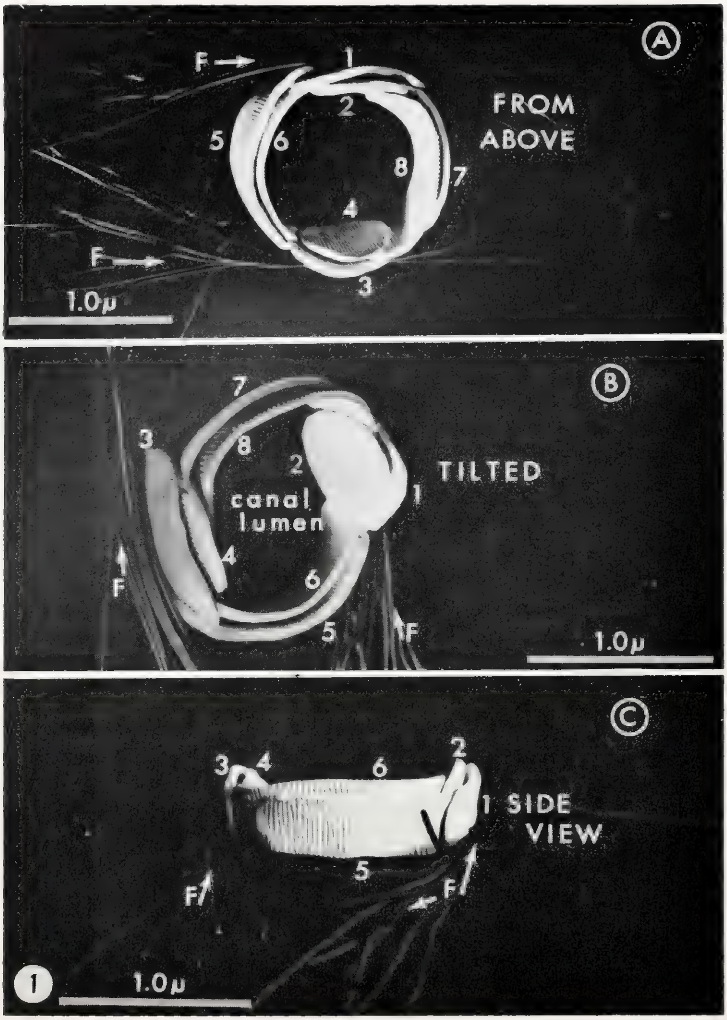


FIGURE 1. Photographs of the Polyform model of the system of semiannular leaves which surround a ring canal of *Habrobracon juglandis*. The model has been photographed after being tilted in various planes. The ring is made up of paired sets of short, broad leaves (1, 2 and 3, 4) and long, narrow leaves (5, 6 and 7, 8). Each of the eight leaves is made up of a parallel array of short microtubules. These are represented by parallel grooves carved into the outer surface of the Polyform leaves. Filaments (F), represented by bundles of insulated wires, extend into the cytoplasm in both directions from the short broad leaves. The filaments extending from the long narrow leaves are not included in the model.

Maraglas and polymerized according to the method of Erlandson (1964). One block was selected for sectioning because of the advantageous orientation of the germarium. Approximately 200 serial, longitudinal sections, each about 70 mμ thick, were cut using an LKB Ultrotome III equipped with a glass knife. Sections were picked up in groups of 8–10 upon Formvar-carbon coated, one-hole copper grids using the LKB section collector-stereoscope assembly. The sections were stained by successive immersions in saturated, aqueous solutions of uranyl acetate (10 min) and lead citrate (5 min) according to the procedure of Frasca and Parks (1965).

Approximately 970 electron micrographs were taken with a Hitachi HU 11A microscope operated at 50 KV. Composite electron micrographs were made from the overlapping prints representing areas from each section at a magnification of

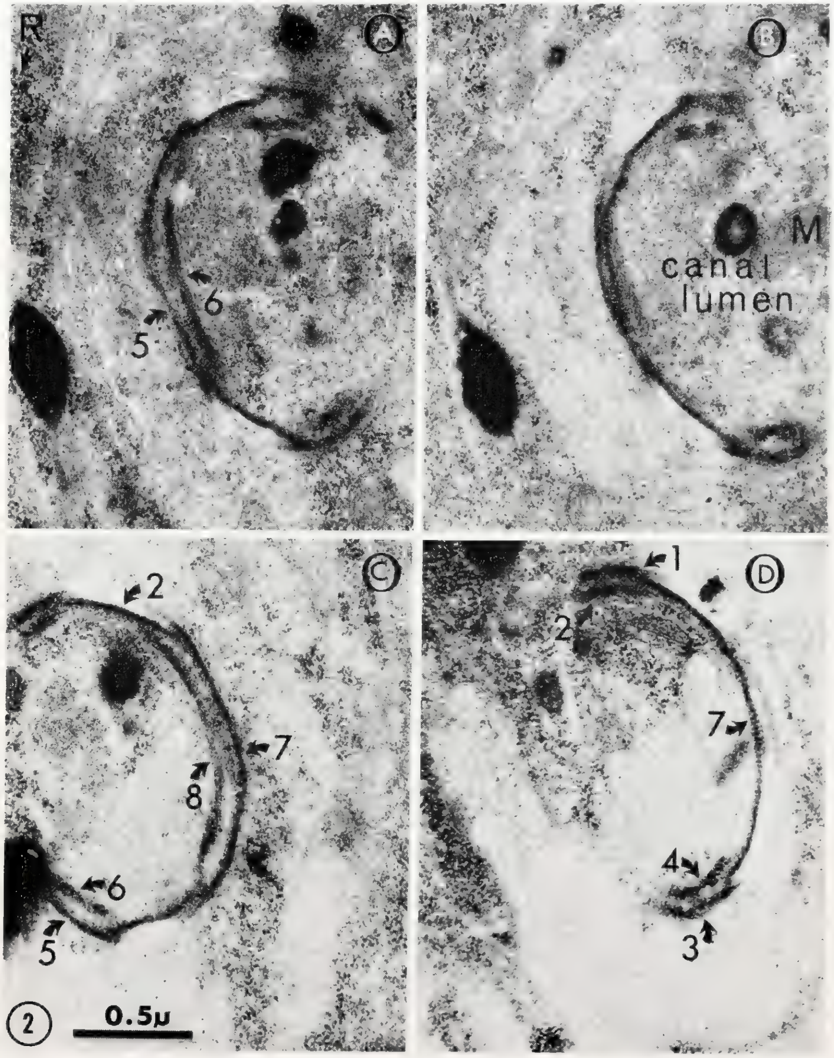


FIGURE 2. Electron micrographs of four serial thin sections from a ring canal found in a cluster of cystocytes located in the posterior region of the germarium. The leaves are labeled as in Figure 1. The cytoplasm contains ribosomes (R) and mitochondria (M).

11,000 ×. The interconnections between cystocytes were localized in 156 such composites. Transversely-sectioned ring canals were observed on rare occasions, and three such canals from cystocyte clusters located within the anterior, middle and posterior portions of the germarium were chosen for study at higher magnification. The appropriate negatives were selected, and enlargements were made using Kodak positive sheet film. The result was a series of positive transparencies showing the canals at 38,000 ×. The transparencies from serial sections were oriented one above the other and viewed simultaneously in order to grasp the three-dimensional morphology of the canal rim. Eventually a 50,000 × model was made of a canal rim using the malleable plastic, Polyform (see Koch and King, 1969 for the details of Polyform model construction).

Results and Conclusions

Each canal connecting sister cystocytes in the *Habrobracon germarium* is in the shape of a laterally flattened right cylinder with an altitude ranging between 0.4 and 0.7 μ (Fig. 1). When viewed in a perfect transverse section the canal from which the model was constructed formed an ellipse with major and minor axes of 1.3 and 1.0 μ, respectively (Fig. 2). The rim surrounding each canal was made up of eight leaves arranged in four pairs. The leaves of each pair overlapped with the leaves of adjacent pairs. The arrangement of the leaves is easily grasped after viewing Figure 1. When the ring is sectioned in the longitudinal or frontal plane one obtains the image seen in Figure 3.

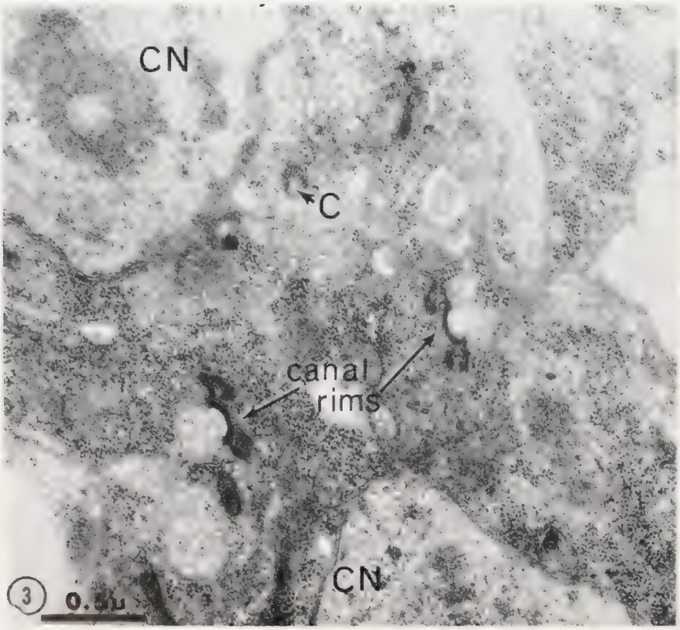


FIGURE 3. A canal rim cut in longitudinal (or frontal) section. The vast majority of the canal rims seen in electron micrographs are sectioned in this manner: C, centriole; CN, nuclei of sister cystocytes.

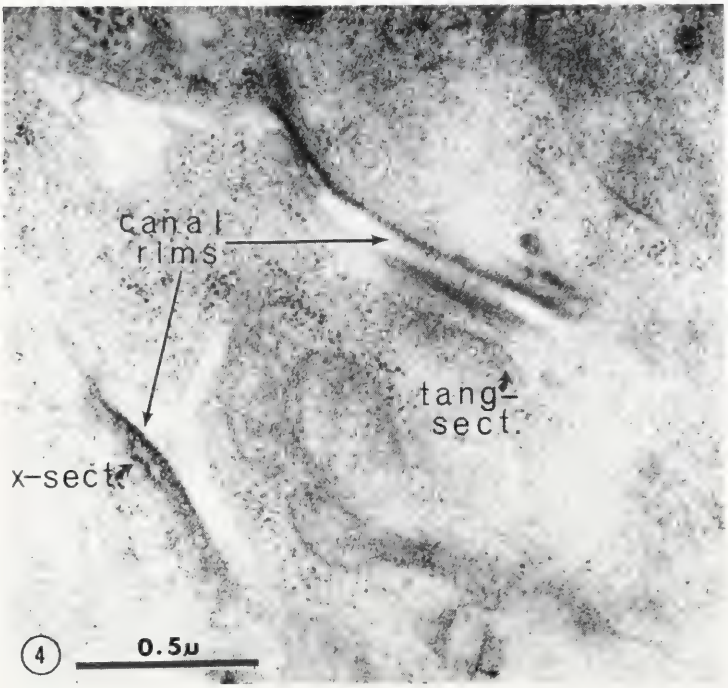


FIGURE 4. Two canal rims; one cross-sectioned, the other cut tangenitally. The parallel orientation of the microtubules in a leaf can be seen in the upper rim.

The short broad leaves have a length of ~1.1 μ and a width of ~0.7 μ. The long narrow leaves have a length of ~2.0 μ and width of ~0.4 μ. The short and long leaves are ~0.05 and 0.04 μ thick, respectively. Each leaf is made up of a monolayer of short, parallel microtubules. These are clearly seen in Figures 4 and 5. The mean outer diameter of each microtubule is ~20 mμ, and each is separated from its neighbor by a space ~10 mμ wide. From the above dimensions we calculate each short and long leaf contain about 40 and 70 microtubules, respectively. These are embedded in an electron-dense matrix.

In Figures 6 and 7 drawings are presented of two cross-sectioned canals. Both are magnified to the same degree, but one has a greater circumference. The dilation has been accompanied by a change in the orientation of the long, narrow leaves. Obviously leaves 5 and 7 have slid apart from leaves 6 and 8. The pairing pattern of the short, broad leaves seems unchanged.

Filamentous attachments are also observed in the cytoplasm, surrounding the leaves of the rim. These filaments are much longer than those embedded in the leaves, but their diameters are about the same. The orientations of these membranes are shown in Figure 8. They seem to attach to all the leaves, but the short, broad leaves seem to possess denser clusters of these cytoplasmic filaments. An electron micrograph illustrating these filamentous attachments lying perpendicular to the rows of microtubules in a canal rim, is presented in Figure 9.

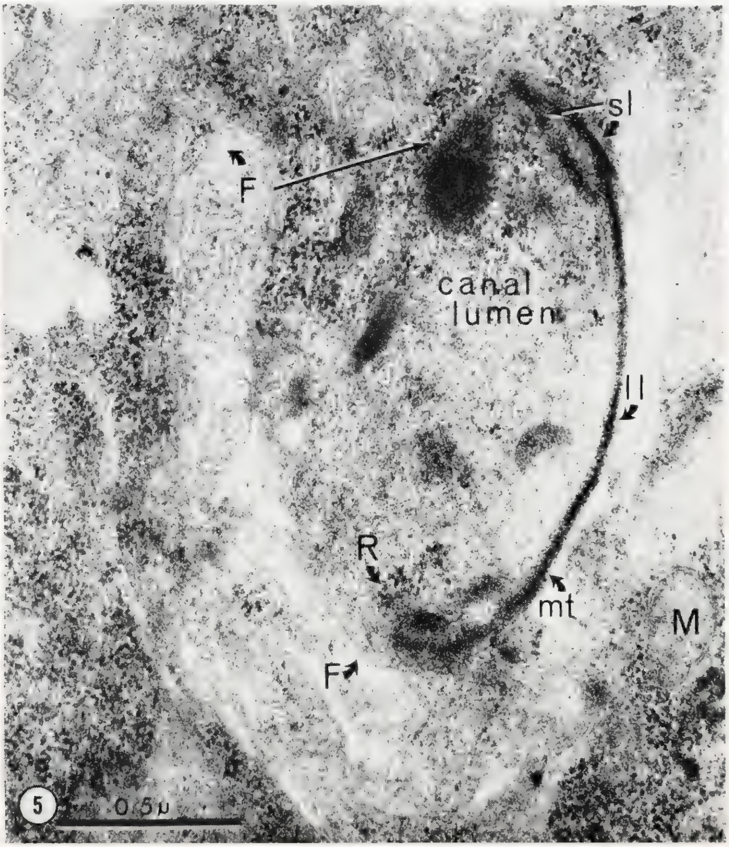
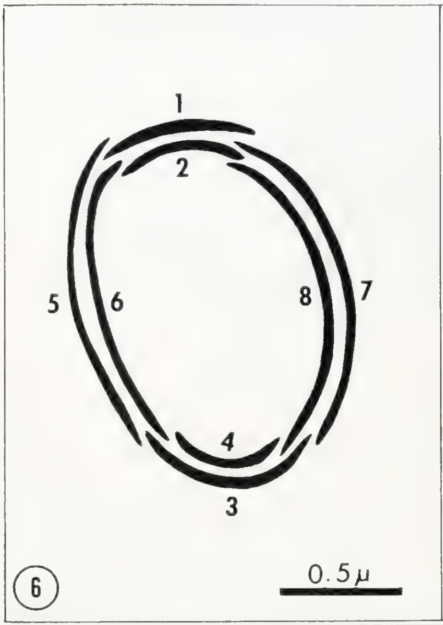


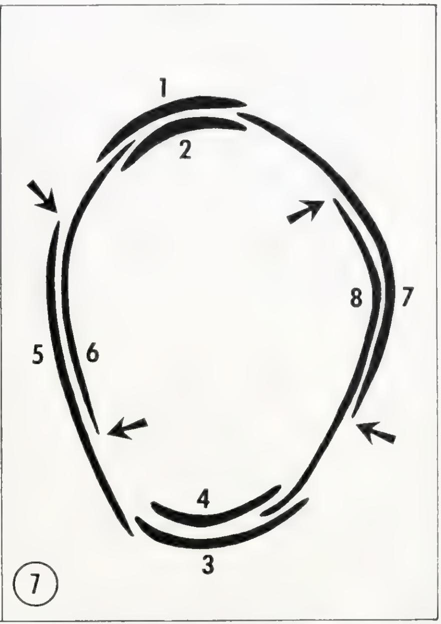
FIGURE 5. Detail of the array of microtubules (mt) in a long leaf (ll) intersecting two shorter leaves (sl) cut in cross-section; F, cytoplasmic filamentous attachments to the canal leaves; R, ribosomes; M, mitochondrion.

DISCUSSION

Koch and King (1969) have shown in *Drosophila melanogaster* that the ring canal rim is completed *prior* to the appearance of the plasma membrane to which the canal rim is later attached. Koch and King suggest that the canal rim is somehow derived from a "mid-body." This is a dense, disc-shaped structure which is commonly observed in the middle of the ephemeral cytoplasmic bridge that connects sister cells in late telophase. In other species ultrastructural studies have shown that the mid-body consists of a disc of electron-dense material in which a bundle of microtubules is embedded (Buck and Tisdale, 1962; Allenspach and Roth, 1967; Rosai *et al.,* 1969). The finding of microtubules embedded in the leaves surrounding the ring canals of *Habrobracon* strengthens the hypothesis that the rim elements are derived from a mid-body.

Koch and King's electron micrographs demonstrate that in *Drosophila* after the ring is formed there is produced along the plane of division a plaque of intercon-





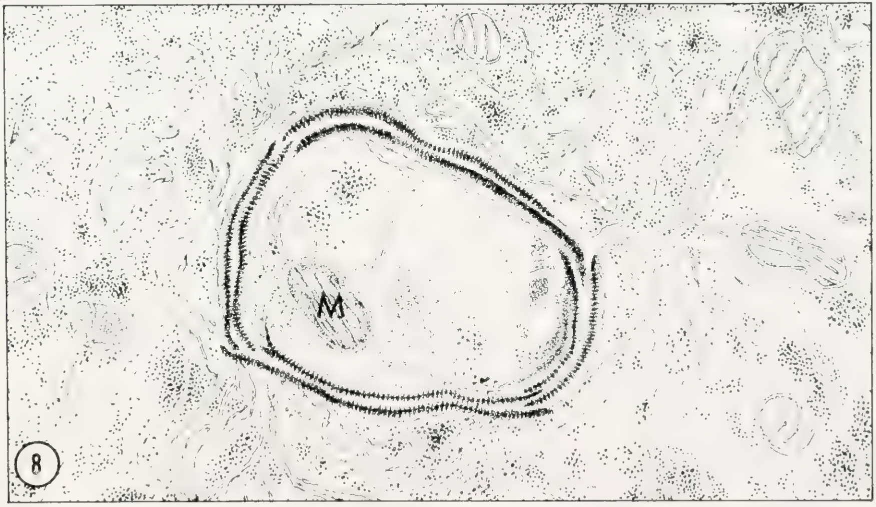


FIGURE 6. An outline drawing of a cross-sectioned canal showing the orientation of the eight leaves which form the rim.

FIGURE 7. An outline drawing of a cross-sectioned canal which is dilated relative to the canal shown in Figure 6.

FIGURE 8. A drawing illustrating the orientation of the cytoplasmic filamentous attachments which surround the canal relative to the microtubules comprising the leaves; M, mitochondrion.

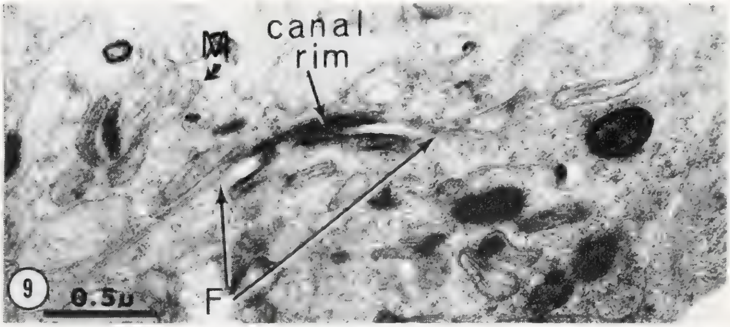


FIGURE 9. An electron micrograph of a glancing section through the upper portion of the canal rim containing short leaves. Cytoplasmic filaments (F) extend in both directions from the surface of the leaves; M, mitochondrion.

nected vesicles and tubules and that these later coalesce to form continuous sheets of membrane which segregate the cytoplasms of the sister cells, except in the region enclosed by the ring. As development proceeds the cystocytes increase in volume, and each canal dilates, its rim becomes thicker, and the inner circumference of the rim becomes coated with a thick deposit having different cytochemical properties than the rim itself. These findings support the hypothesis that in *Drosophila* the canal rim is a metabolically active organelle capable of undergoing morphological changes of functional significance.

In *Habrobracon* it is also obvious that each canal rim dilates as the cystocytes develop. The mechanism of dilation seems to involve the sliding apart of specific paired leaves. A decision as to whether or not the attached cytoplasmic filaments play any role in this movement must await further investigations.

Canals similar to those connecting insect cystocytes have been found between young oocytes in the brine shrimp, *Artemia salina* (Anteunis, Fautrez-Firelefyn, and Fautrez, 1966), between the oocyte and its accessory cells in the polychaete, *Diopatra cuprea* (Anderson and Huebner, 1968), and between oocytes in the ovary of the rat (Franchi and Mandl, 1962), the hamster (Weakley, 1967), the rabbit (Zamboni and Gondos, 1968), and the mouse (Ruby, Dyer and Skalko, 1969). In the case of rodents and many other higher mammals a huge majority of the early oocytes ultimately degenerate. Davidson (1968) has suggested that, as in the meroistic system of insects, most of the interconnected oocytes actually function as nurse cells and degenerate once this task is accomplished. Thus throughout the animal kingdom during oogenesis sister germ cells are commonly found to be connected by cytoplasmic canals at an early stage in their development. Time will tell as to whether dilatable canal rims of the type described here are restricted to a few insect species or are widespread throughout the animal kingdom.

The authors are grateful to Drs. Elizabeth Koch and Michael Cummings for their valuable suggestions with regard to three-dimensional reconstruction techniques and electron microscopy of serial sections, to E. John Pfiffner for preparation of the Polyform model and the inked drawings, and to Misses Catherine Tomaseski and Susan Grober for their conscientious assistance. This research was supported by grants from the U. S. Public Health Service (5TIGM903–7) and the National Science Foundation (GB 7457).

SUMMARY

The canals connecting the ovarian cystocytes of *Habrobracon juglandis* are enclosed by a ring made up of eight leaves. Each leaf is composed of a monolayer of between 40 and 70 short parallel microtubules which may be derived from the mid-body. The sliding of certain leaves past one another allows the ring to dilate as the cystocyte grows.

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OSMOREGULATION OF *CRANGON SEPTEMSPINOSA* SAY (CRUSTACEA: CARIDEA)[1]

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Species of estuarine Crustacea are subjected to an environment in which fluctuations in salinity may be sudden or continuous. Other factors, such as temperature and dissolved oxygen, will normally vary with salinity. Any one or more of these factors may influence the process of osmotic regulation (Kinne, 1964). Although a causal relationship may be expected to exist among these different factors, this has not as yet been fully explained (Lange, 1968).

Numerous osmoregulatory studies have been performed on a variety of penaeid and palaemonid Crustacea. Panikkar (1941) observed that *Palaemonetes varians*, *Palaemon serratus* (= *Leander serratus*) and *Palaemon elegans* (= *L. squilla*) were capable of hypotonic regulation in normal sea water and of hypertonic regulation in diluted media. Similar regulatory patterns have been reported for *Metapanaeus monoceros* (Panikkar and Viswanathan, 1948), *M. dobsoni*, *Penaeus indicus*, *P. carinatus* (Panikkar, 1951), *Palaemonetes intermedius* (Dobkin and Manning, 1964), *Penaeus aztecus*, *P. duorarum* (Williams, 1960), and *Palaemon macrodactylus* (Born, 1968).

Considerable attention has been given to the caridean, *Crangon crangon* L., a euryhaline species which ranges from the White Sea (Wollebaek, 1908) to the Moroccan coast of Africa (Nouvel and Panouse, 1965). Caudri (1937) provided evidence that the osmoregulatory capacity of *C. crangon* is related to temperature. Mathias (1938) observed moderate survival to low salinities with death ensuing after 7–8 hours of exposure to freshwater. Investigations by Broekema (1941) indicated that optimum salinity varied with age of the specimens and that hypo- and hypertonic osmoregulation was more efficient at higher temperatures and inhibited at low temperatures. Lloyd and Yonge (1947), in field and laboratory observations, concluded that males cannot withstand as low salinities as females and that optimal salinity, at 15° C, is higher for males. Flügel (1960, 1963) showed that osmotic resistance to low and high salinities (1–5‰ and 30–90‰) is higher at 5° C than at 15° and 20° C. The efficiency of osmoregulation does, however, decrease at temperatures below 5° C. This loss of resistance is correlated with the fact that in the northern Baltic Sea, *C. crangon* is incapable of survival in low salinity water at temperatures near freezing.

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Crangon septemspinosa Say is a good example of a euryplastic inhabitant of Atlantic east coast estuaries. Within its geographic range from the Gulf of St. Lawrence (Squires, 1965) to eastern Florida (Rathbun, 1929; Whitely, 1948; Williams, 1965), it has been collected within temperature and salinity ranges of −3° to 25° C and 3.4 to 32‰, respectively (Stickney, 1959; Price, 1962; Haefner, P. A., Jr., unpublished). A recent multivariate study (Haefner, 1969) has delineated the influence of temperature, salinity and temperature-salinity interaction on survival of this species. The present study was inaugurated in order to determine if *C. septemspinosa* does regulate its internal salinity, and if so, to what extent under the influence of a variety of temperature and salinity combinations.

METHODS

Shrimp used in this research were collected at Lamoine Beach, Maine, and transported in natural seawater of 29–31‰ to experimental facilities. After thermal acclimation, the shrimp were transferred to 30‰ seawater and maintained on a mixed diet of haddock, brine shrimp and blue mussel. A holding time of at least 7 days, and usually longer, was adhered to prior to subjecting the shrimp to experimental conditions.

The various salinities used in the research were made with commercially available synthetic sea salts (Segedi and Kelly, 1964) and determined by the low precision method of Strickland and Parsons (1965). The pH was monitored with a portable Beckman meter.

Temperature was maintained in a constant temperature room and monitored with a Taylor recording thermometer.

Freezing points of blood and seawater were determined with a thermoelectric cryoscope (Clifton Technical Physics, Wannamassa, New Jersey) reading in centigrade degrees. After the animal was blotted dry with filter paper, a blood sample was obtained by inserting a micropipet into the pericardial cavity accessible through the membrane between the posterior edge of the carapace and the first abdominal segment. Hemolymph was drawn into the oil-filled pipet and two 1-nanoliter volumes were transferred to the cryoscope platform for reading. Samples of seawater were handled in a similar fashion.

Survival studies on *C. septemspinosa* indicated that definitive mortality occurred by the fourth day when the shrimp were subjected to changes in salinity (Haefner, 1969). In an effort to determine the time involved for the surviving organism to equilibrate osmotically with its experimental environment, and the extent of the steady state, the following experimental routine was performed. Shrimp were transferred from the holding aquarium water (30‰) to experimental seawater of 15‰, at 5° C. Freezing point determinations were made on the blood of at least 9 individuals of each sex (juvenile, male, non-ovigerous female) selected prior to transfer and at 1, 2, 4, 8, 12, 18, 24, 48 and 96 hours after exposure to the new salinity. After this initial pattern was observed, the number of sampling times was reduced for the transfer of shrimp from 30‰ to 45‰, at 5° C.

The major part of this study was relegated to analysis of freezing point depressions of shrimp subjected to 5 salinities (15, 25, 30, 35, 45‰) and 2 temperatures (5, 15° C) for periods greater than 96 hours. Extensive mortality prevented the collection of sufficient data for 5‰ even when a stepwise salinity transfer was employed.

The freezing point depression data were treated according to Tan and Van Engel's (1966) modification of the graphic method of Hubbs and Hubbs (1953). Such a chart indicates if there are significant differences between the means of juvenile, male and female shrimp for each temperature-salinity combination as well as differences between the shrimp subjected to different salinities and for different periods of time. Significant difference in the means at the 1% level occurs if the solid, cross-hatched or stippled bars ($t_{0.05} \times$ standard error on either side of the mean) do not overlap one standard deviation on either side of the mean (hollow bars). Significant difference at the 5% level occurs if the patterned bars overlap without reaching one of the means. There is no difference in the means if the patterned bars overlap the means.

RESULTS

Experimental acclimation times

Sand shrimp, transferred acutely from one salinity to another, experience changes in the osmoconcentration of the hemolymph which are related to the osmotic strength of the receiving media. Although the trend is a gradual change in osmoconcentration toward that of the new salinity, there was a noticeable oscillation in the pattern prior to the attainment of a new steady state.

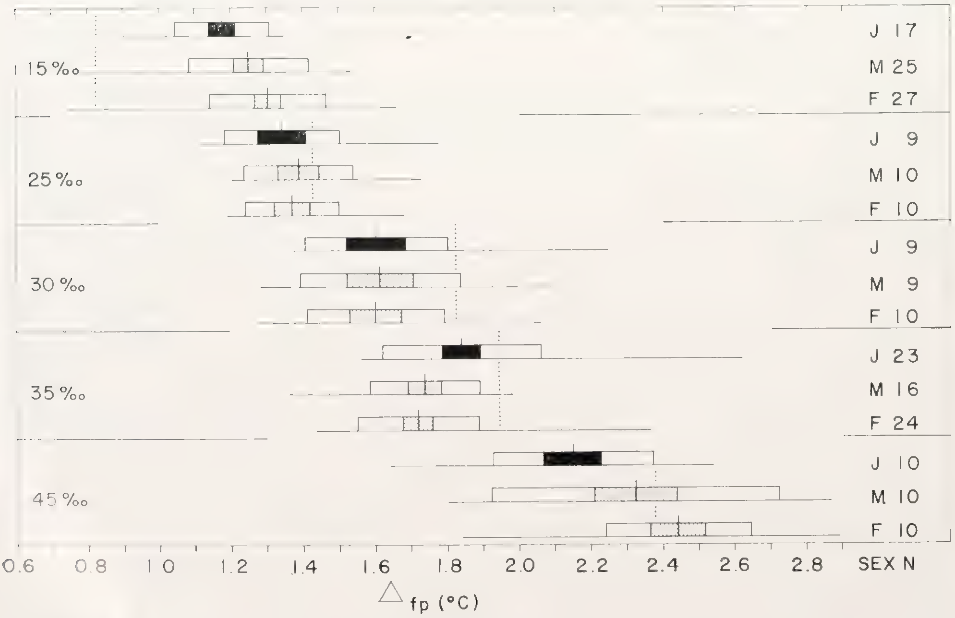

FIGURE 1. The means, standard deviations and $t_{0.05}$ standard errors for freezing point depressions of blood of juvenile (J), male (M) and female (F) sand shrimp exposed to six salinities for longer than 4 days at 5° C. Number (N) of shrimp sampled is indicated in right margin. Dotted lines indicate mean freezing point depressions of 5 salinities.

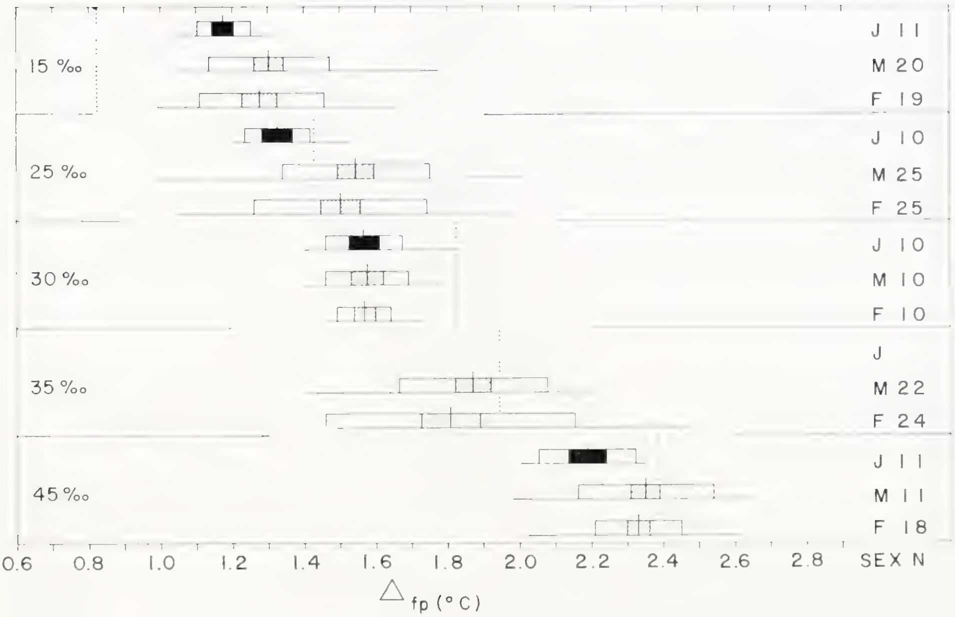

FIGURE 2. The means, standard deviations and $t_{0.05}$ standard errors for freezing point depressions of blood of juvenile (J), male (M) and female (F) sand shrimp exposed to five salinities for longer than 4 days at 15° C. Number (N) of shrimp sampled is indicated in right margin. Dotted lines indicate mean freezing point depressions of 5 salinities.

Within 8 hours after the transfer from 30‰ to 15‰, juveniles showed less resistance to the change than did adult shrimp as their blood osmoconcentration decreased immediately after transfer. Adult blood concentration increased initially to a level higher than that of the acclimation seawater and then gradually decreased. A period of oscillation followed until a new steady state was reached at $\geq$96 hours. The magnitude of the range, standard deviation and standard error for each group oscillated throughout the sampling period with an observed maximum variability at 8, 8 and 12 hours for juveniles, males and females, respectively.

In the transfer from 30‰ to 45‰, all shrimp showed a gradual increase in blood osmoconcentration. Juveniles exhibited an over-compensation at 24-hours prior to hyperregulation observed at $\geq$48 hours. Male shrimp were hyperregulating at 24 hours but their blood osmoconcentration gradually diminished to an isotonic state relative to the external medium. Females were not observed to hyperregulate until 48 hours. This condition was maintained in excess of 96 hours.

Total osmotic concentration

Shrimp at 5° C exhibit varying degrees of osmoregulation in response to a wide range of salinity (Fig. 1). At 25‰ there are no differences among the means of juvenile, male and female shrimp but blood osmoconcentration of adults is isotonic to the external medium while that of juveniles is slightly hyposmotic. Although there is regulation of a hyposmotic state in 30‰, there are again no significant differences among means of the shrimp.

There is a 1% to 5% difference between the mean blood concentration of juvenile shrimp which is hypotonic to seawater of 35‰, and that of adult shrimp which exhibit a more effective hyposmotic regulation. There is no significant difference between males and females at this salinity. At 45‰, juveniles are hypotonic to the environment while adult shrimp are in an isosmotic state. Differences of 1 and 5% exist between the mean osmoconcentration of juveniles and that of the male and female shrimp, respectively.

Osmoregulation at 5° C is most obvious at 15‰ salinity, in which the blood concentration is maintained hypertonic to the external media. Adults regulate better than juveniles (1–5% difference) and females regulate significantly higher than males (5% difference) in this salinity.

The situation at 15° C (Fig. 2) is similar to that described above for 5° C. At 25‰, blood concentrations of adults (no difference between male and female) are hypertonic to that of the seawater and significantly different (1%) from that of juvenile shrimp which remain hypotonic to the environment.

All components of the population (no differences among means) are hypotonic at 30‰. Juveniles maintain hypotonicity at 45‰ but the internal environment of adult shrimp approaches isotonicity in 35‰ and is isosmotic at 45‰. No differences between the means of adults occurs at each of these salinities. In 15‰, males and females (no difference between mans) hyperregulate to a greater extent (1% difference) than do juveniles.

Temperature effects

There is no apparent or significant difference (see Figs. 1, 2) between the osmoregulatory response of juvenile shrimp at 5° and 15° C. The blood osmoconcentration, relative to the external medium, is hypertonic at 15‰, and hypotonic at salinities $\geq$ 25‰, regardless of the temperature.

Male shrimp hyperregulate more effectively at the higher temperature (5% difference at 15‰; 1% at 25‰; see Figs. 1, 2). In higher salinities (30–35‰), they tend to hyporegulate better at the lower temperature (1% difference at 35‰; see Figs. 1, 2).

Females are more hyperosmotic in 25‰ salinity at the warmer temperature (1% difference; see Figs. 1, 2), and they hyporegulate more effectively in cooler, 35‰ water (5% difference; Figs. 1, 2).

DISCUSSION

Crangon septemspinosa has been shown to exercise sufficient control over its internal osmoconcentration to the extent that it is hyposmotic in normal seawater and hyperosmotic in diluted seawater. As such, it resembles other euryhaline Caridea as well as other crustacean groups such as amphipods, isopods, panaeid shrimp and grapsoid and xanthid crabs (Robertson, 1960). In this category, the sand shrimp is distinct from those marine and brackish-water Crustacea which are isosmotic in normal seawater and hyperosmotic in diluted media, and from those forms known as osmoconformers (Kinne, 1963).

Certain variations in the regulatory response to osmotic stress, which have been attributed to differences in age, size, sex and stage of life cycle for other forms

(Flügel, 1960; Williams, 1960; Kinne, 1963, 1964), are exhibited by *C. septemspinosa.* A generalization made from studies of the response to high salinities by decapods normally living in fresh water is that almost all types eventually become isosmotic with salinities appreciably higher than the level at which blood concentration is regulated in fresh water (Born, 1968). A similar response is exhibited by adult *C. septemspinosa* to salinities greater than that of normal seawater. Hyposmotic regulation in adult *C. septemspinosa* failed at 45‰ at both temperatures. Although the response of *C. crangon* to 45‰ was not tested, there were indications that the blood concentration was approaching isotonicity at 40‰ (Flügel, 1960, 1963). The failure of adult *C. septemspinosa* to regulate at these salinities can be correlated with the lethargic behavior observed at the time of sampling and the experimental mortality (Haefner, 1969). This apparent loss of hyporegulatory capacity was not observed for the juvenile forms. The different response noted between juvenile and adult shrimp in this situation may indicate that different regulatory mechanisms, such as those outlined by Born (1968), are employed.

Flügel (1960) determined that adult *C. crangon,* at 5° and 15° C, was isotonic at 27–28‰, hypertonic from 3–26‰ and hypotonic from 30–40‰. This pattern was corroborated by Hegemann (1964). Juvenile specimens of *C. septemspinosa,* at 5° and 15° C, exhibit iostonicity at lower salinity (22–23‰) than adults (25‰) at 5° C. At 15° C the isotonic point is higher (27–28‰) for adults. In this pattern and in the fact that no significant differences in the mean osmoconcentrations between male and female shrimp (except at 15‰ at 5° C) were noted, *C. septemspinosa* is similar to *C. crangon.*

The equilibration time is similar for the two species. Flügel (1960) observed that a 5-day period of adaptation was adequate for *C. crangon* as differences ($\Delta_i - \Delta_o$) reached a maximum at 3 days and leveled off at 5–7 days. Survival (Haefner, 1969) and freezing point data (Figs. 1, 2) for *C. septemspinosa* indicate that a time period ≥ 4 days is satisfactory for this species to reach a new steady state. Other Crustacea (Williams, 1960; Thompson and Pritchard, 1969) have been shown to achieve a new steady state in less time, but lack of an adequate number of examples prohibits any meaningful comparison of species at this time.

The pattern of maintaining higher blood concentrations at lower temperatures, generally exhibited by brackish-water crustaceans (Robertson, 1960) is not adhered to by *C. septemspinosa.* There is no apparent influence of temperature on juvenile forms but adults exhibit higher blood concentrations at warmer temperatures. They are, in effect, regulating more effectively in diluted seawater and less effectively in normal seawater at warmer temperatures (15° C).

Although the osmoregulatory pattern of *C. septemspinosa* seems unusual, it can be related to its biology and migratory behavior within the estuarine zone. In Maine waters, sand shrimp spend most of the year in deeper, cooler, more saline water. Thus they are subjected to salinities of 30–34‰ at lower temperatures (5° C), the experimental combination which invokes the most effective hyposmotic regulation. They appear in the shallow estuarine zone when the water temperature reaches 5–6° C, migrate into mesohaline (3–17‰) waters, and remain there as long as prevailing water temperatures do not exceed 18° C. Under these conditions the more efficient hyperosmotic regulation is operable.

The results are similar to those obtained for *Penaeus aztecus* and *P. duorarum*, in which a loss of osmoregulatory ability occurred with lowering temperature (Williams, 1960). In the penaeids, however, the juveniles experienced the same loss as the adults.

The results do not corroborate those of Flügel (1960), who observed that osmoregulatory performance in *C. crangon*, measured as $\Delta_i - \Delta_o$, was more effective in animals adapted to 5° C than those adapted to 15° C. *Crangon septemspinosa* hyperregulates better in dilute seawater (<25‰) and hyporegulates more effectively in higher salinities at 5° C rather than at 15° C.

The difference in osmoregulatory response to temperature between the two species of *Crangon* may be related to their temperature-salinity tolerance. Haefner (1969) compared the mortality of *C. septemspinosa* with data available for *C. crangon* (Broekema, 1941) and observed that the former species is more tolerant of a given salinity range at higher temperatures than is the latter. Conversely, *C. crangon* exhibited greater survival at lower temperatures. The differences noted are probably related to the geographic ranges of the species in question. The European *C. crangon* extends farther north and not nearly as far south as *C. septemspinosa*. In their respective temperature regimes, each species would be suitably adapted to migrating into the diluted estuarine zone: *C. crangon* can do this most effectively in cooler climates while *C. septemspinosa* is better suited for accomplishing this feat in warmer waters.

The author wishes to acknowledge Mrs. Patti Kruse and Mrs. Peggy Martin for their assistance in the collection of data pertinent to this study.

SUMMARY

1. Freezing point determinations were made on the blood of *Crangon septemspinosa* exposed to five salinities (15, 25, 30, 35, 45‰), at two temperatures (5, 15° C).

2. Sand shrimp, transferred acutely from 30‰ to either 15‰ or 45‰, experienced changes in hemolymph osmoconcentration which were related to the osmotic strength of the receiving medium. A time period of about 4 days was required for the shrimp to reach a new steady state at 5° C.

3. *Crangon septemspinosa* was observed to regulate its internal osmoconcentration to the extent that it was hyposmotic in normal seawater (30–35‰) and hyperosmotic in diluted seawater (15–25‰). In 45‰, adults were isosmotic but juveniles remained hyposmotic to the external medium.

4. There was no apparent temperature influence on the regulatory pattern of juvenile shrimp. Adults, however, exhibited high blood concentrations at 15° C than at 5° C. They were, in effect, regulating more effectively in diluted seawater and less effectively in normal seawater at the warmer temperature.

5. Osmoregulatory performance of *C. septemspinosa* was compared with that of the European species, *C. crangon*. Differences in the response to temperature between the species were discussed in terms of their geographic ranges and related temperature regimes.

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SUBUNIT COORDINATION IN THE FIREFLY LIGHT ORGAN[1]

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An interesting type of cellular integration is the synchronous coordination of responses of many effector units to produce a brief pulse which is the summation of the activities of the individual units. Some neuroeffector activities, such as the discharge of electroplaques of electric fish (Albe-Fessard, 1961) are remarkably well synchronized; the duration of activity of the entire organ is comparable to that of a single unit.

Another effector system which exhibits coordination of single units is the firefly light organ. In the genus *Photuris,* there are two light organs located on the ventral portions of abdominal segments 6 and 7, each of which contains about 1000 light emitting cells ("photocytes"). These are grouped into organizational units of 10–16 photocytes in a rosette pattern around a central core ("cylinder") which contains the trachea and nerves. One to four "rosettes" may be stacked vertically through the thickness of the organ. Nerve trunks from segmental ganglia branch to each cylinder and terminate in complex "end organs" near the photocytes (see Buck, 1948; and Smith, 1963; for further histological details).

Neural control of light production has been amply demonstrated (Buck and Case, 1961; Case and Buck, 1963; Buck, Case, Hanson, 1963; and Magni, 1967). Data from localized stimulation of small portions of the light organ (Hanson, 1962) suggest that the physiological unit of control peripheral to the ganglion may be the portion of the light organ supplied by a nerve branch. Visual observations by Buck (1966) indicate that under some experimental conditions, responses can be elicited from smaller units, perhaps the rosettes and individual photocytes. Thus, some ambiguity exists as to the size of the smallest physiological unit which is coordinated in a flash of the light organ.

The normal degree of unit synchrony in the firefly organ is such that the onset of activity appears simultaneous and uniform over the entire surface when observed by the gross methods of low power microscopy or photomultiplier viewing of several limited portions of the tissue (Buck, Case, and Hanson, 1963). As Buck (1966) points out, however, it has not yet been determined whether a whole-organ flash consists of well-synchronized subunit responses, each having kinetics similar to the organ flash or whether it is an integration of staggered briefer responses of the individual units. If the latter is indicated, as the data of Buck (1966) suggest, then identification of the single unit and an analysis of the spatial and temporal distribution of unit responses will add to present knowledge of the excitation mechanism of firefly luminescence.

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With image intensification tubes (Reynolds, 1968), it has become possible to follow the events in the firefly flash using cinematographic techniques with substantial optical magnification. Consequently, we undertook the following study to obtain further information concerning the participation and coordination of unit responses in the firefly effector system.

Materials and Methods

The fireflies used were adults of *Photuris* sp. (probably *versicolor* or *lucicrescens*) collected near Princeton, New Jersey or raised from larvae collected near Bethesda, Maryland.

The firefly was placed in a slot milled into a lucite microscope slide and secured dorsal side down. Restrained in this manner, the firefly flashed either spontaneously or after mild irritation. For some experiments, all or part of the light organ was divested of cuticle by cutting along the edges of the segment and peeling away the cuticle with sharpened forceps. A transient glow emanated from the exposed area. This glow was prolonged for photography by injection of norepinephrine into the abdomen. Scintillation was induced in some animals by abdominal injection of 10^{-3} *M* eserine, an anticholinesterase (*cf.* Case and Buck, 1963).

The slide holding the firefly was placed on the stage of a compound microscope from which the ocular lens had been removed. Light from the firefly was then projected in focus on to an image intensifier tube (English Electric Valve Co., type P-839) operated at gains of 10^3 to 10^6. Using this system, cinematography was possible at up to 64 frames/sec where exposures of 5–10 sec would have

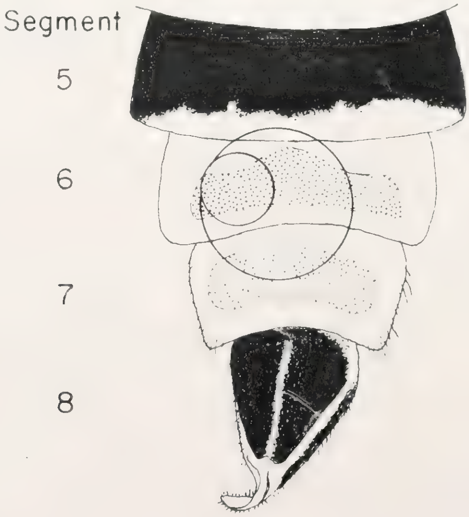

FIGURE 1. Ventral view of the posterior abdomen of a female *Photuris sp.* locating approximately the image intensifier fields for Figure 2 (large circle) and Figure 3 (small circle). Fields shown in Figures 4 and 7 are of the size but not necessarily the location illustrated by small circle.

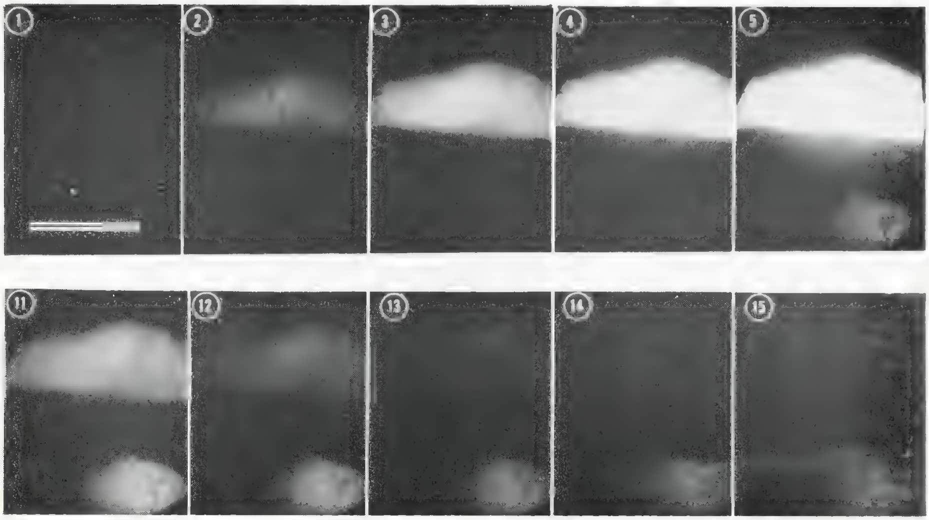

FIGURE 2. Coordination of the two segmental light organs. The two segments sometimes light asynchronously, as illustrated here. Time between frames is 16 ± 2 msec. Only frames 1–5 and 11–15 of a 17-frame flash are shown. The anterior organ turns on 2–3 frames (32–48 msec) ahead of the posterior. Calibration bar = 1 mm. For orientation of the field of the firefly, see Figure 1 (large circle).

been required using conventional optics (Reynolds, 1968). The image tube has an input cathode one inch in diameter and the electron optics result in a magnification of approximately 0.95. The tube is capable of resolving 25 line pairs per millimeter at the input. The dark current (or noise) introduced by the intensifier consists mainly of single electrons emitted from the cathode surface, which result in bright spots of light approximately 40 microns in diameter on the output phosphor. However, about 5% of the noise spots are significantly larger than this and result from multiple electron emissions from the cathode due to ion bombardment. Thus, when the signal consists of nearly circular spots, it is important that optical magnification in the system occur before the intensifier cathode. In the present work this magnification (3×, 20×, and 100×) was accomplished by the objectives of the microscope.

The anode was photographed with a 16 mm Bolex movie camera at 32 or 64 frames/sec. Calibration of the shutter cycle of the camera indicated considerable variability; however, periods of constant shutter speed occurred when the camera was neither accelerating after being turned on nor decelerating as the spring neared its unwound limit. In such periods the cycling interval was 16 ± 2 msec at "64 frames/sec" and 32 ± 4 msec at "32 frames/sec." All records were selected from such periods of uniform shutter speed in the middle of a run.

The positions of the image intensifier field relative to the light organ of the firefly at the lower magnifications are illustrated in Figure 1. Several types of luminescent activity were photographed; normal flashing, dim flashing, scintillation, and glowing. For dim flashes and for scintillation, enlarged positives were made from the cinefilm and the spots of light from the firefly organ were identified and traced from frame to frame on a transparent overlay. Thus, the appearance of each spot could be followed as the flash waxed and waned. In the dim flashes, the spots were followed from flash to flash as well as from frame to frame. A long sequence of submaximal flashes was filmed from one animal, but only those in best focus (flash numbers 1–3 and 17–19) were analyzed. The interval between these two series was about two minutes.

To determine the sizes of the microsources, the diameter of each spot in flashes 17–19 was measured. Calibration was accomplished using photographs of a micrometer slide mounted in place of the firefly and viewed through the same optical system.

The sizes of the photocytes, cylinders, and rosettes were determined from photographs of standard histological sections of *Photuris* sp. cut 10 μ thick in an orientation parallel to the ventral surface. Tissues were fixed in Navaschin fixative and stained with Delafield's hematoxalin and eosin. These photographs were supplied by John Buck and Miriam McLean of the National Institutes of Health (see acknowledgments).

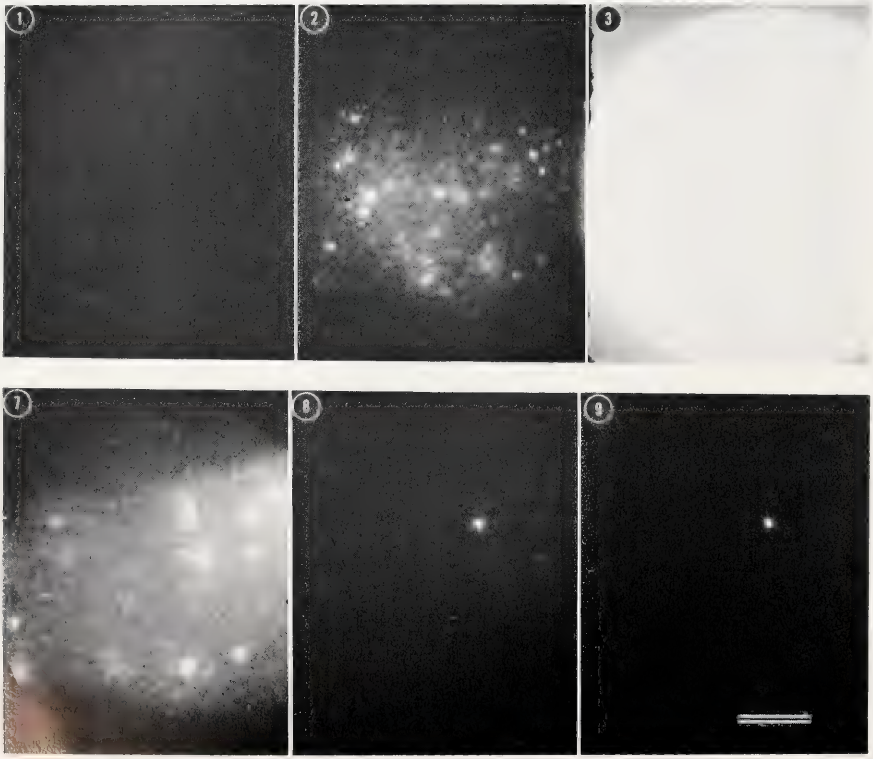

FIGURE 3. Coordination of microsources in a normal flash. Some sources precede and others lag the rest of the organ. Only frames 1–3 and 7–9 of an 11-frame flash are shown. Frame 3 is overexposed due to the flash of the entire organ. Three frames identical to frame No. 3 have been omitted between rows. Somwhat more than $\frac{1}{3}$ of the sixth-segment's light organ is shown (see Fig. 1, small circle). Calibration bar is $200\ \mu$. Time between frames is 32 ± 4 msec.

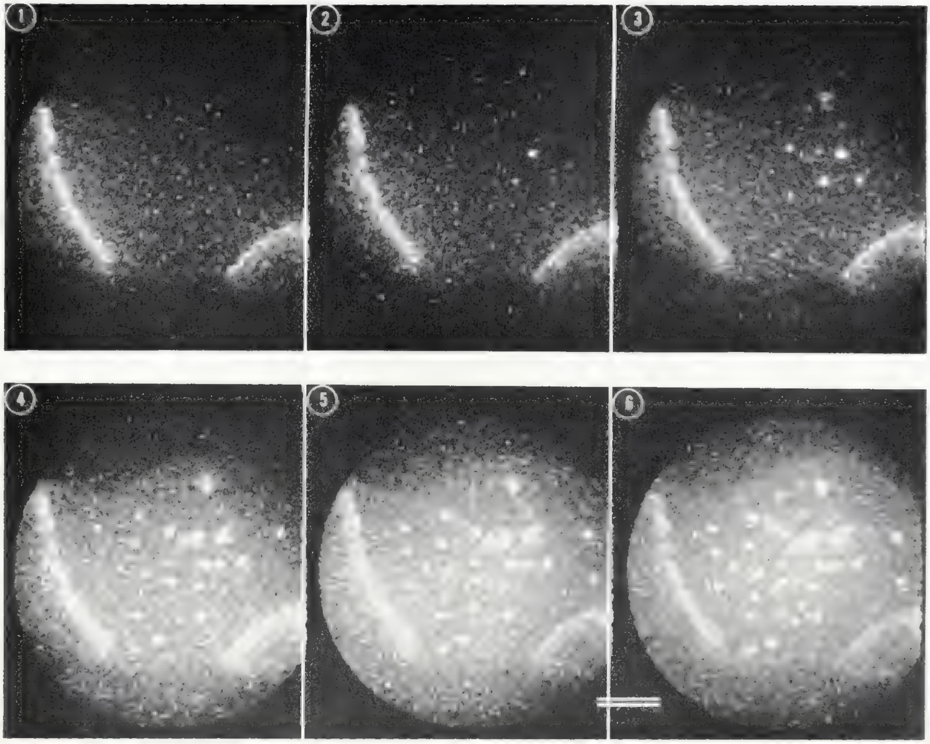

FIGURE 4. Coordination of microsources in a submaximal flash. Frames 1–6 of flash No. 18 in a series of filmed submaximal flashes. Note that spots remain in constant relative positions through several frames. Diagonal light bands are tissues glowing because of incisions through the cuticle overlying the light organs. These bands serve as markers for registration of the transparent overlay used in identifying spots on successive frames. The differences between tube noise spots and microsource spots are easily observed in frames 1 and 2. Time between frames is 16 ± 2 msec. Calibration bar is 200 μ.

Where temporal information was not required, superimposition prints were prepared by sequentially projecting several frames of the negative cinema film on the same photographic paper at reduced intensities which eliminated most of the noise spots. This procedure was followed for Figures 8, 9, 10b, 12, and 13.

RESULTS

I. *Coordination of gross areas of organs*

The extent of temporal coordination between the two light organs of the firefly has been examined with the image intensifier system. Results show that usually both organs flash coincidentally (within 16 msec); however, occasionally the anterior or posterior segment may lead. Figure 2 shows a filmstrip taken at 64 frames per second with low optical magnification in which the anterior segment leads the posterior segment by 2–3 frames, or 32–48 msec (see Fig. 1 for orientation of the field on the firefly).

Lack of complete synchrony can also be seen within a single organ. In Figure 3, individual spots (frame 2) precede the activity of the rest of the organ (frame 3). The decay phase is also asynchronous, with small areas of activity lingering on well past the extinction of the others.

II. *Coordination of Microsources*

Normally when an organ flash is observed, the details of single unit activity are not seen due to lack of contrast. However, fireflies occasionally emit submaximal flashes consisting of small spots of light scattered across the otherwise dark organ (Fig. 4). These microsources are of nearly uniform size and appear similar in size and shape to the spots which lead and lag the organ flash (Fig. 3). Thus, microsources appear to be functional units and are here tentatively proposed as the smallest such physiological unit of the firefly light organ.

TABLE I

Microsource durations

Type of light emission	N	Mean duration of microsources ± S.E.	Range	Organ flash duration (approx.)
*Individual flashes:				
Flash 2	101	151 ± 6 msec	60–340 msec	340 msec
Flash 3	55	156 ± 8	31–360	400
Flash 17	85	147 ± 7	31–310	425
Flash 18	80	112 ± 6	47–250	300
Flash 19	109	124 ± 5	31–250	320
**Pooled flashes	430	139 ± 3	31–360	
***Scintillations	129	81 ± 2	31–156	

* Listed are the durations of the microsources as well as of the total flashes which they comprise. N is the number of microsources. Organ flash durations are only approximate, since the end points were estimated by comparison with a normal flash form.

** Microsource data from flashes 2, 3, 17, 18, and 19 were lumped for these calculations.

*** Scintillation data were obtained from 160 consecutive frames of cinema film. All statistics and ranges were calculated from original data and do not include uncertainties of absolute time calibrations, which is approximately ± 15% (see Materials and Methods).

Spatial and temporal diestributions of the microsources within a single flash. Assuming that submaximal flashes are essentially the same as normal flashes except for a reduction in number of participating microsources, cinematographic recordings of the light organ during this reduced activity provide information concerning the participation of unit sources in the organ flash. A record taken at 64 frames/sec demonstrates asynchrony of microsources (Fig. 4). The temporal patterns provided by the sequential appearances of the spot do not correspond to gross morphological structures, such as innervation (Hanson, 1962). However, the spatial distribution of the spots results in a distinct pattern which allows individual sources to be identified in successive frames throughout a flash as well as in successive flashes.

The temporal distribution of microsources throughout a flash is illustrated in Figure 5. This figure shows that individual spots do not remain lit for as long as does the overall organ flash, and that the duration of the latter is partly the result of asynchronous initiation of the former.

The durations of activity of the microsources vary from 31–360 msec (S. D. = 61) and are normally distributed about a mean of 139 msec (see Table I).

Only rarely did a spot appear to extinguish and then revive within a single flash. Only five such cases out of 430 occurred, with an average of 32 msec "off-time." These data suggest that a long recovery period is not required for

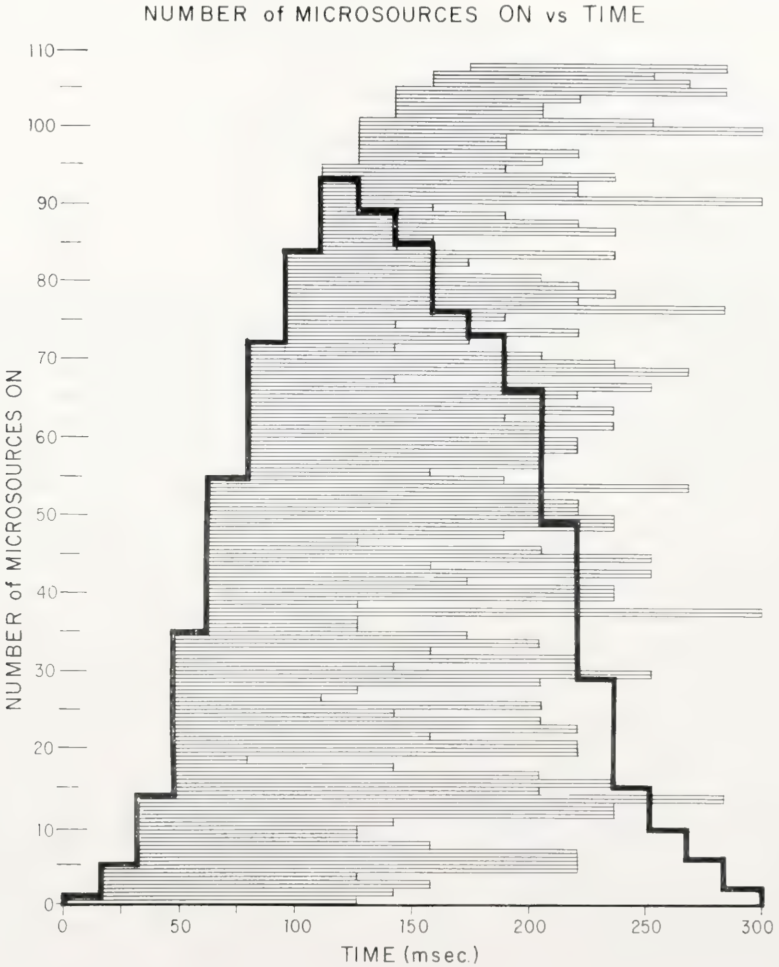

FIGURE 5. Microsource durations in a single flash (No. 19). Each microsource numbered on the ordinate according to order of appearance is represented as a bar (consisting of a line and two spaces) stretching from the time of its appearance to the time of extinction. Vertical steps at left illustrate the number of new microsources appearing in successive frames. The heavy line indicates the total number of microsources visible at any one time.

TABLE II

Decrement of microsource lifetimes during the course of a flash

	Fraction of flash				
	First $\frac{1}{3}$		Second $\frac{1}{3}$		Final $\frac{1}{3}$
* Mean duration ± S.E.	173 ± 5 (msec)		143 ± 4		101 ± 4
N	145		144		141
P of difference		<0.001		<0.001	

* The total number of spots measured in 5 submaximal flashes (see Table I) were categorized according to the fraction of the flash in which it was first observed. The means of the durations of all spots in each category were than calculated.

these microsources, or that the microsource is a compound source. Since the light organ has depth, the latter is a possibility.

Spatial and temporal distributions of microsources in successive flashes The spatial pattern of lighting microsources is well reproduced in successive flashes: of the spots which are active in one flash, an average of 76% also appear in the next flash.

The relative temporal order in which the microsources appear in successive flashes also tends to repeat; that is, the order of appearance of the individual spots in four out of five flashes correlated significantly with the order of appearance of the succeeding flashes. This is also reflected in the uniformity of the latencies

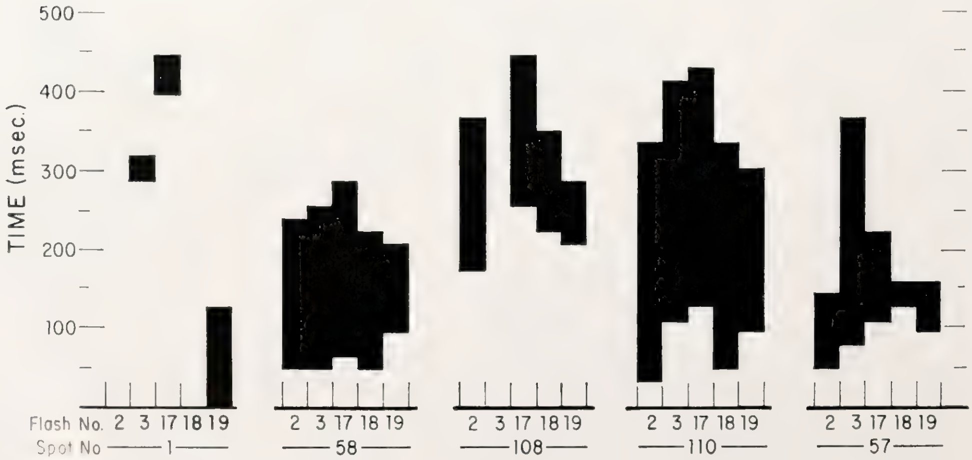

FIGURE 6. Microsource durations in successive flashes *vs.* time after appearance of first microsource. The vertical bars represent the active period of a microsource relative to the beginning of the flash. Horizontal repetition of bars represents the activity of the same microsource in the flashes numbered on the abscissa. The spots shown were specifically selected to illustrate variations in the following response parameters: delay relative to earliest microsource, duration, and dependability of appearance. For example, spot No. 1 appeared only in flash Nos. 3, 17, and 19 with a great deal of variability in delay and duration, whereas No. 58 appeared in all flashes and was quite consistent.

(time between initiation of organ flash and onset of spot activity) from flash to flash of four of the five individual microsources depicted in Figure 6.

Another temporal relationship observed is that between the duration of a microsource and its time of appearance. The correlation in this case is significantly negative ($r = -0.48$, $p < 0.001$), indicating that the earlier spots last longer than those appearing later. This is also illustrated by Table II: responses appearing in the first $\frac{1}{3}$ of the flash last longer than those which comprise the remaining portions of the flash.

III. *Participation of microsources in scintillation*

Scintillation consists of apparently asynchronous activity of minute areas of the light organ which occurs occasionally in normal animals and can be evoked artificially by injection of neurally active drugs, such as eserine. Serial photographs of the light organ during the eserine-induced scintillation shown in Figure 7 provide an indication of this type of activity. The spots of light are similar in appearance to the microsources, and data were obtained in the same manner as in the dim flashes.

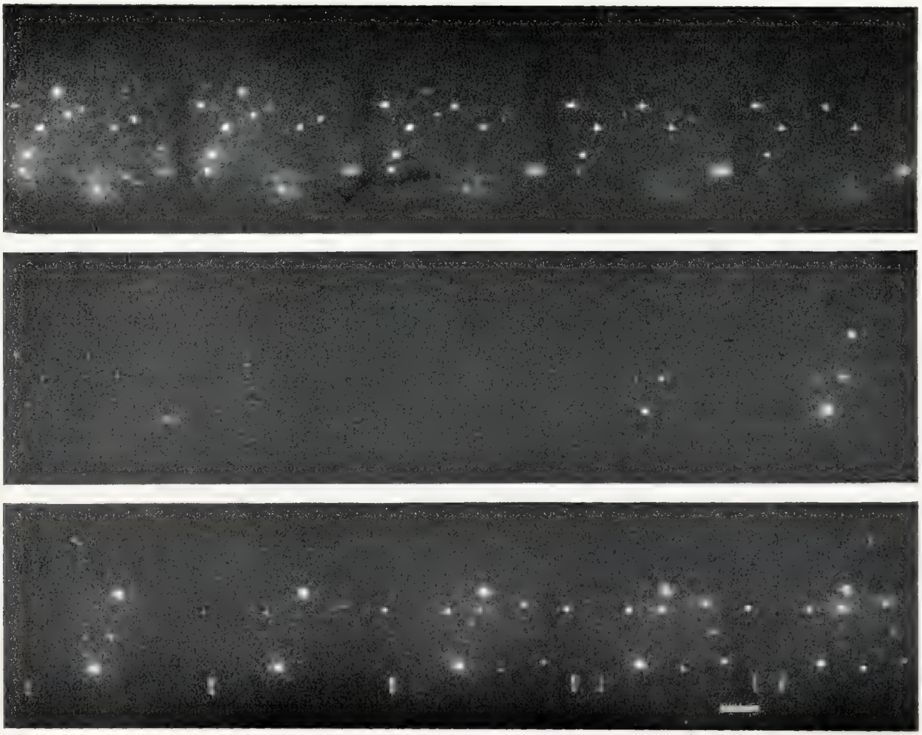

FIGURE 7. Scintillation. A series of 15 consecutive cinema frames taken shortly after injection of 10^{-3} *M* eserine into the abdomen. Note that spots stay in register for several frames. Entire circular field represents about $\frac{1}{3}$ of one segment (see Fig. 1). Time between frames is 16 ± 2 msec. Calibration bar is 200 μ.

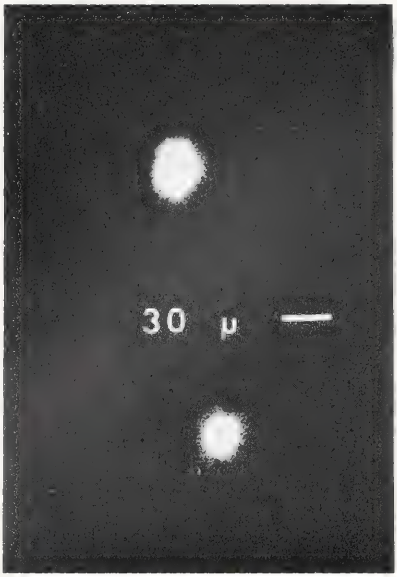

FIGURE 8. Size and shape of individual microsources. The upper is slightly elliptical, 39 × 30 μ. The lower is nearly circular with a diameter of 26 μ. This figure is a highly enlarged print of a frame from a scintillation series.

The durations of scintillation spots are less than those of flash spots, averaging 81 msec (range 31–156 msec, S.D. = 25, see Table I). No repeatable spatial patterns of the spots were observed.

When observing eserine-induced scintillation, the visual impression is of random activity and it has been described as such in the literature. However, statistical treatment of the temporal data (autocorrelation and Fourier analysis) from one animal showed significant ($p < 0.001$) periods of increased microsource activity at 170 and 1370 msec intervals.

IV. *Shape and size of the microsource*

The usual microsource spot appears round in the photographs (Figs. 4, 7, and 8) although about 10% were ellipses having major: minor axis ratios of 1.3–2.2. Occasional spots are larger and very elongate, and thus are probably aggregates of microsources (Fig. 9).

The sizes of the spots composing submaximal flashes range from 11 to 36 μ with a mean diameter of 21.6 ± 0.3 μ (S.E.), as documented in Table III. The scintillation spots were similar in size, averaging 20.7 μ (range 10–36 μ). The pre- and post-flash spots also fall into this range, averaging 31 μ in diameter. The sizes of the spots were not significantly different from flash to flash, or from flashes to scintillation (Table III).

The diameter of a microsource appears to be quite constant throughout its lifetime, *i.e.*, over the several (average = 8.9) continuous cinema frames on which it is visible. The sizes of the spots in the first and last frames during an appearance

TABLE III

Apparent microsource size during flashes and scintillation

	Number of measurable spots	Mean* diameter ± S.E.	Range
Flash 17	63	21.6 ± 0.8 μ	11–36 μ
Flash 18	70	22.1 ± 0.5 μ	15–34 μ
Flash 19	63	21.0 ± 0.5 μ	15–30 μ
Lumped 17, 18, 19	196	21.6 ± 0.3 μ	11–36 μ
Scintillation	123	20.7 ± 0.4 μ	10–36 μ

* Mean diameters of microsources were determined by measuring the spots in every frame in which a spot appeared. No significant differences between means were detected between any categories.

are 8% smaller than the mean size, but this could be a photographic artifact due to the changing brightness.

DISCUSSION

Identification of the unit source

The foregoing descriptions of the activities of unit sources are independent of their anatomical identity. However, in view of the extensive data on microsource diameter (Table III), such an identification can now be made.

To aid in such identification, a tangential histological section of a light organ is illustrated together with an image intensfied photo of a glowing organ in Figure 10 and a composite diagram of histological features and dimensions (Fig. 11). The histological section (Fig. 10a) shows that the photocytes fan

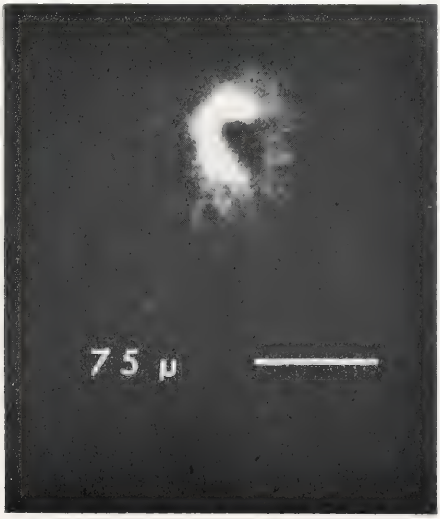

FIGURE 9. Aggregation of microsources. From the same scintillation series as Figure 8. Such aggregations comprised less than 1% of the scintillation spots.

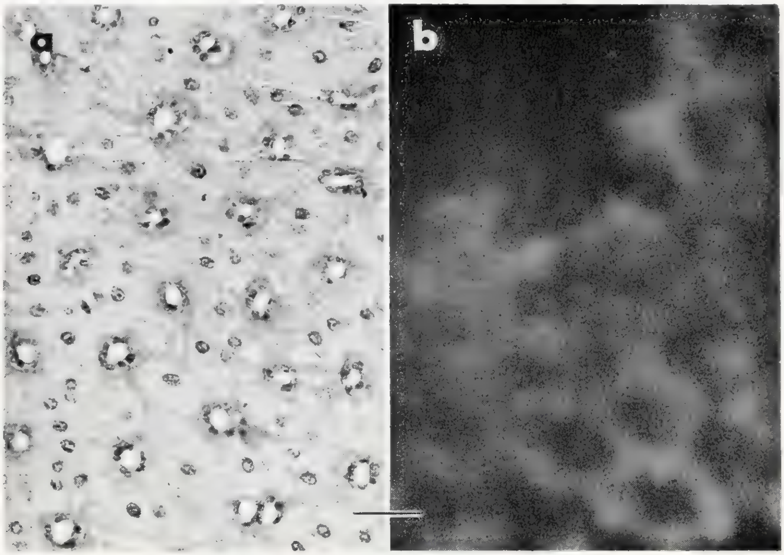

FIGURE 10. (a): Histological section of the light organ of *Photuris*, supplied by M. McLean (see acknowledgments). Tangential section (parallel with ventral surface of the light organ). Open circles *ca.* 10 μ in diameter are trachea cut in cross section; dense bodies immediately surrounding them are the "tracheal end organs" (tracheal end cells, tracheolar cell bodies, nerve endings) as well as the packed mitochrondria in the cortical zone portion of the photocytes adjacent to the tracheal end organs. The "cylinders" average 14 μ in diameter and comprise trachea and end organs. The photocytes radiate out from the cylinders, stretching the entire distance, an average of 34 μ, from the rim of one cylinder to the rim of its nearest neighbor. The darkly stained nuclei of many photocytes appear in this section midway between cylinders. Tracheoles emerging from the tracheal end organs are seen coursing between photocytes in upper right. Center-to-center distances between nearest cylinders range from 30 to 75 μ. (b): Glowing organ at same magnification as 10a. The nearest spacing of dark centers averages 50 μ (range 35–60 μ), which is comparable to cylinder nearest spacing in 10a. Calibration bar is 50 μ.

out in a rosette pattern from the axial "cylinders", which comprise trachea (open circles) plus the tracheal end organs (tracheal end cells, tracheolar cell bodies, nerve endings) seen here as darkly stained nuclei immediately surrounding the trachea. Measurements from several such sections indicate that the average spacing between neighboring cylinders is 42 μ (range 30–75 μ) which compares favorably with 52 μ (range 35–60 μ) for the spacing of the dark centers of the glowing rings in Figure 10b. Thus from geometrical considerations it is clear that the portions of the cylinder which emit light are the more centrifugal elements, namely the photocytes.

Further, the dimensional data illustrate that only part of any photocyte emits light; the width of the bright band in Figure 10b (average: 25μ, range: 20–31 μ) is less than the length of the photocytes in Figure 10a (average: 34 μ, range: 23–50 μ). Furthermore, the dark centers of the glowing rings in Figure 10b appear larger (diameter 25–40 μ) than the diameter of the cylinders (10–17μ) in Figure 10a. Therefore, the dark centers must include the cylinder plus some adjacent portion of the photocytes. The non-luminous portion of the

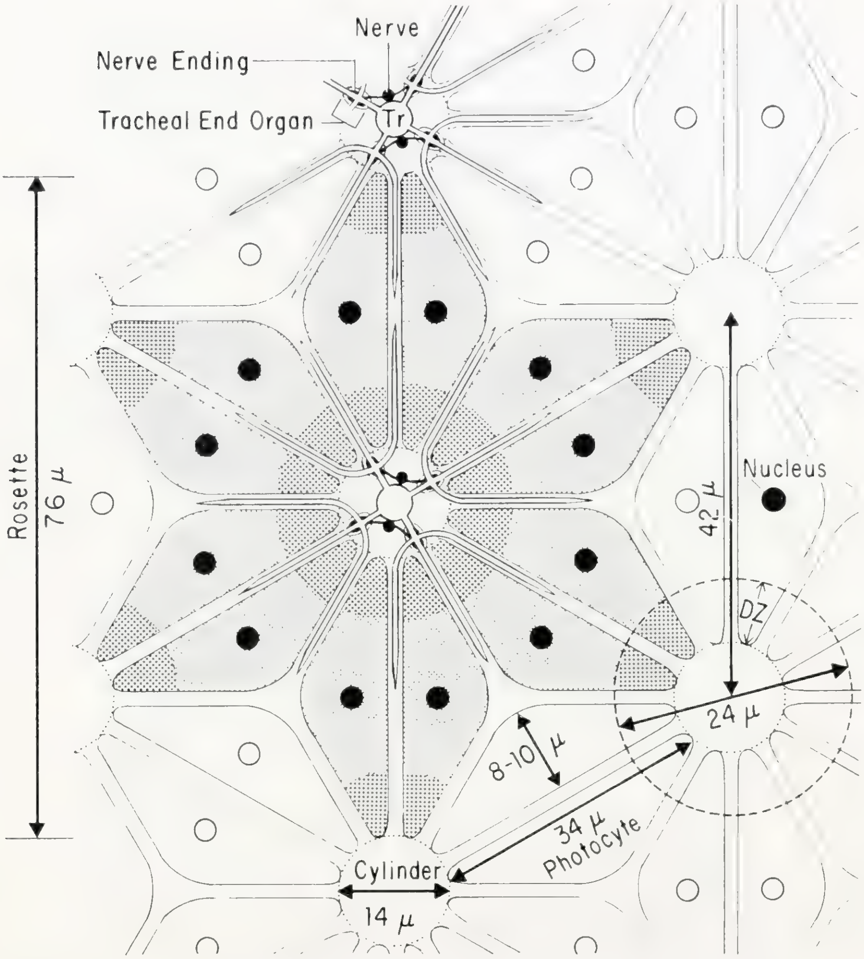
Nerve
Nerve Ending
Tracheal End Organ
Tr
Rosette
76 μ
Nucleus
42 μ
DZ
24 μ
8-10 μ
34 μ
Photocyte
Cylinder
14 μ

FIGURE 11. Composite diagram drawn from Figure 10a; dimensions refer to means of measurements and thus may not be strictly additive.

photocyte would correspond well with the "differentiated zone" of the peripheral cytoplasm of the photocytes, which lacks the "photocyte granules" so abundant in the central region (see review by Buck, 1948; Beams and Anderson, 1955; Smith, 1963).

It is also evident in Figure 10a and 11 that the diameter of a full rosette of photocytes would be the length of two photocytes plus the cylinder, or 82 μ. Measurements of several such rosettes range from 60–90 μ with an average of 76 μ. The sizes of full glowing rings seen in Figure 10b would be expected to correspond with this anatomical rosette minus the differentiated zones, or about 66 μ. Such measurements of glowing rings average 72 μ in diameter.

Figure 12 is a higher magnification of a portion of a skinned organ showing two complete glowing rosettes. This figure more graphically illustrates the dark centers 25–40 μ in diameter, surrounded by glowing elipses 15–30 μ long and 7–9 μ wide, some of which are separated by a thin dark line (arrows).

Further evidence that the microsource is smaller than the rosette is seen in Figure 13, in which glowing rosettes and single flash spots of a submaximal flash occur simultaneously in the same organ.

In the above context, the unit source which produces the flashspots and scintillation spots can now be identified. The average diameter of this microsource (21.6 μ) is somewhat less than the average length of a photocyte (34 μ) but is within the width (20–31 μ) of the light band surrounding the dark cylinders in Figure 10b. If only the central portion of a photocyte emits light, as mentioned earlier, a 21.6 μ spot could be accounted for. However, since the width of the photocyte is 6–10 μ, activity confined to a single cell would result in an elongate spot with a major: minor axis rato of from 2 to 5. Yet, only about 10% of the microsources were observed as ellipses, with elongation ratios of 1.3 to 2.2 (average 1.6).

In view of the foregoing, a composite picture emerges which identifies the functional unit source as being the *central portions of two or three adjacent photocytes.*

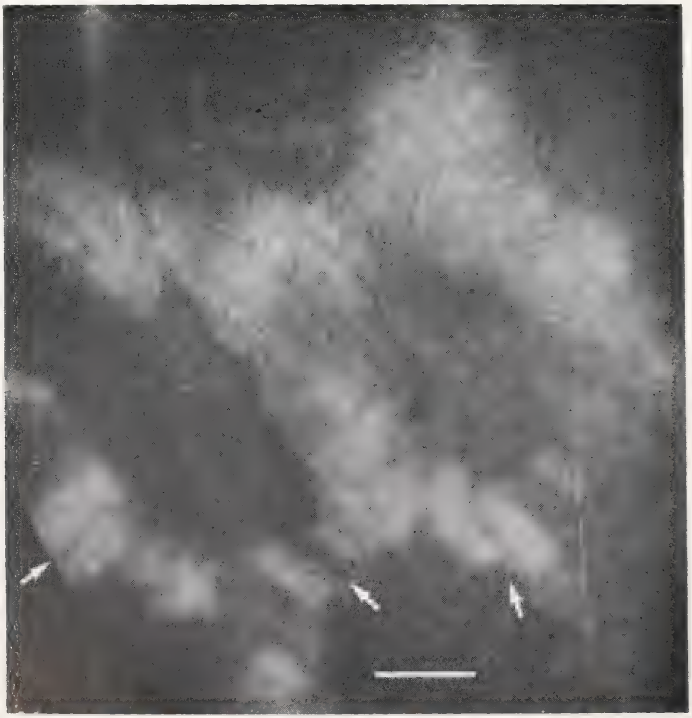

FIGURE 12. High magnification of a glowing light organ. Two complete rosettes are shown along with portions of adjacent ones. A number of light emitting areas which are about the size and shape of individual photocytes (7 × 21 μ) can be seen, with some pairs of photocytes separated by a thin dark line (arrows). Calibration bar is 20 μ.

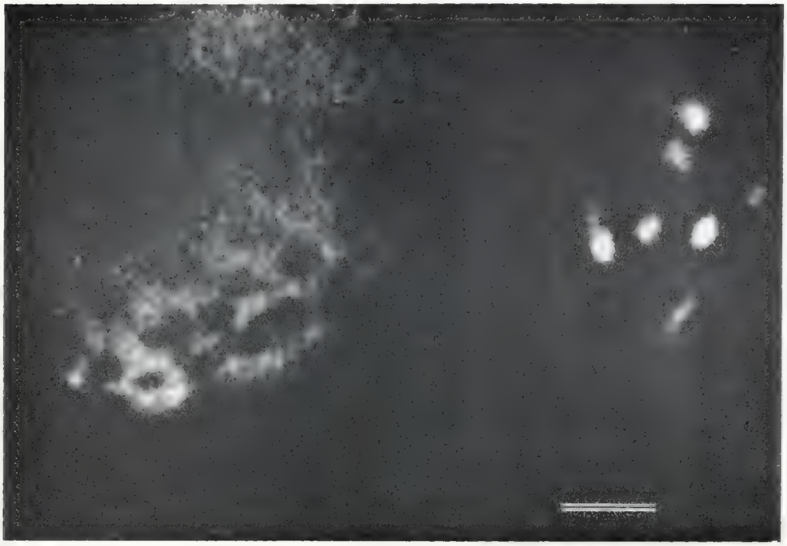

FIGURE 13. Glow and flash spots in the same organ. Glowing area (left) appeared after removal of the cuticle from half of the light organ. Microsources appear in a submaximal flash in the unskinned portion of the same light organ. Sizes of the spots range from 20 to 40 μ; diameters of the light rings (glowing rosettes) average about 70 μ. Calibration bar is 100 μ.

That the 21.6 μ microsource is actually a physiological unit is supported by the appearance of such a light source in three different conditions: (1) before and after an organ flash (Fig. 3); (2) in submaximal flashes (as discrete units; Fig. 4); and (3) during scintillation (Figs. 7, 8).

Previous workers in the field have generally considered the rosette to be the smallest physiological functional unit, perhaps partly because the histological data show photocytes highly organized around cylinders which act as distribution points for tracheae and nerves. However, Buck (1966) reported that luminous units of two sizes were observed visually: "glowing rings," and smaller "points" which compose the rings. Buck further reported that the glowing rings tend to be involved in the normal, widespread and coordinated responses, whereas disorganized, local and frenetic excitation patterns include an increasing proportion of the smaller units. Extending Buck's observations, the data in the present paper indicate that microsources are activated individually, even in normal flashes (Fig. 3). Adjacent microsources may be activated simultaneously to produce elongate spots or parts of rosettes (Fig. 9). Thus, if the activation of microsources making up a rosette were coordinated within the temporal resolution of the eye, the glowing ring would be observed as a functional unit. Buck's observations that this is the *normal* case implies that preferred innervation patterns exist within the ganglion which simultaneously excite all the axons innervating the microsources of a rosette.

The system for the control of light emission by the unit sources has not yet been fully elucidated. The morphological data of Smith (1963) show that the nerve terminates in a complex "end organ" which abuts several (2–4) photocytes. From these end organs, the tracheoles bifurcate or trifurcate and, wrapped in extensions of the tracheolar cell, penetrate between the photocytes. Smith suggested that upon arrival of the nerve impulse, the excitation of the photocytes could occur by (1) the diffusion of neural chemicals through the end cell into neighboring photocytes; or (2) the secondary depolarization of the tracheolar cells which might then be channeled between the photocytes. In either case, a single nerve ending could control the activity of two to four adjacent photocytes. This inference is strongly supported by the physiological demonstration in the present paper that the unit source is two or three photocytes.

Although the data suggest that the photocyte pair (or trio) is the unit of function, it may not be the smallest. Smaller units having very short durations were not rigorously excluded by this study, since spots smaller than 10 μ are not easily differentiated from the noise of the image tube unless the spots recur from frame to frame. Smith (1963) reports photocyte granules *ca.* 2.5 μ in diameter. Such would not have been detected in the present study as discrete units of light emission, particularly if their duration is comparable to a shutter cycle (16 msec.) and thus would have appeared in only one frame. Since Buck (1966) showed photomultiplier recordings of some light emitting sources lasting only 20 msec, this is a possibility.

Coordination of light emission

The observation that the two light organs usually but not invariably light in coincidence confirms similar reports of visual impressions (*e.g.* Buck, 1966). However, the apparent variability of this occurrence (Fig. 2) suggests that separate nerve pathways from the central nervous system control the sixth and seventh segmental light organs.

The lack of complete coordination of the unit sources in producing a mass flash is evident in Figure 3–6. Some variability in synchronization of activation was observed, as flashes with differing rates of rise were observed. Another illustration of the degree of coordination of the microsources is that the durations of the organ flashes are considerably longer than the mean durations of the spots (Table I). Thus, the time spread of the organ flash is partly due to variability in microsource durations (see Table I). In reference to the question posed in the introduction, then, the organ flash is certainly the integration of briefer, staggered responses of a population of units which have activities of variable duration.

The shape of the decay phase of the light emission curve may be due to more than just the lack of new microsources turning on and the decay in intensity of the emitting ones. The fact that the average spot duration decreases with time (Table II) suggests the existence of an active turn-off mechanism. This would be in agreement with the conclusions of Buck and Case (1961) from electrophysiological data. However, another possibility is that units which appear late in the flash receive a lower activation stimulus or have a higher threshold which is manifest as a later onset of activity and a shorter duration.

The recurrence of the same spots from flash to flash indicates that the effectors are capable of activity in each successive flash. This shows that the refractory time for each microsource is no greater than the minimum interflash interval (about 300 msec in this series of spontaneous flashes) and may be considerably less, as suggested by the data that some spots reappear in the same flash after once extinguishing. If so, the same photocytes may participate in each of the triple flashes that is the hallmark of the field behavior of males of some photurids.

Scintillation

In contrast to the partially coordinated responses of microsources making up the dim flashes, the microsources in scintillation appear to "sparkle" throughout the light organ. This phenomenon has been described by Case and Buck (1963) and also Buck (1966) who suggested that scintillation in response to eserine results from periodic "uncoordinated single unit firing". These observations are extended by statistical analyses presented here showing the asynchrony is not complete and that periodicity does occur. Case and Buck (1963) also suggested that these small luminous sources comprise "both single photocytes and small aggregations". This is basically supported by the present work: aggregations are observed (Fig. 9), but single spots (Figs. 7, 8) provide most of the activity. Since scintillation spots are not of significantly different sizes from the flash spots (Table III), the suggestion that each single spot is probably a photocyte pair or trio is applicable to both flashing and scintillation.

The nature of the response of the single unit

Since the organ flash is the combined responses of many individual units, the question arises as to the nature of the contribution of each: do the units respond with a quantal or graded energy output? From the standpoint of comparative physiology, the situation is anologous to muscle: are the single units all-or-none, or graded?

Although not conclusive, the results shown in Tables I and II strongly suggest the latter. The disparity between the durations of the microsources during flashing (139 msec) *vs.* scintillation (81 msec), the marked decrease in duration of the microsources as the flash proceeds, and the differences exhibited by microsources in successive flashes (Fig. 6), all suggest that there is no characteristic quantal response. This corresponds to Buck's (1966) visual observations that the "individual point intensity is not all-or-none but can be graded".

The single unit response, then, is apparently more analogous to the slow muscle fibers than to the all-or-none fast muscle fibers. The firefly evidently controls its light output by modulating single unit response as well as by recruiting additional units. This may provide an explanation for some of the flashing behavior of fireflies in the field: male fireflies often reduce flash intensity when approaching the female in a mating signal exchange.

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SUMMARY

1. Microphotographs were obtained of a firefly (*Photuris sp.*) light organ on cinema film at 64 frames per second with the aid of an image intensifier.

2. Analysis of the film indicates that the two light organs usually flash simultaneously, but cases of one organ leading by 32–48 msec are seen.

3. Asynchrony is also observed within the light organ during a flash. Submaximal flashes are comprised of small spots of light ("microsources") which turn on at various times throughout the flash. Their durations average 139 ± 3 (S.E.) msec (range 31–360 msec) compared with 300–425 msec for an organ flash. Microsources are of fairly uniform size and shape having an average diameter of 21.6 ± 0.3 μ (S.E.) and a range of 11–36 μ. Microsources of this size are also seen as the functional unit of uncoordinated emission displays known as scintillation. It is suggested that the microsource is the smallest physiological unit of function in the firefly light organ.

4. Comparisons of glowing organs with histological sections demonstrate that the center portions of the "rosettes" are dark, and the centrifugal elements of the rosettes (the photocytes) emit light.

5. Microsources (21.6 μ in diameters) are somewhat smaller than the measured length of photocytes (34 μ) but larger than the width (8–10 μ). Thus the functional unit is identified as the center portions of 2 or 3 adjacent photocytes.

6. The whole organ flash is the integration of briefer staggered responses of a population of these single units which have activities of variable duration and intensities.

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GROWTH AND DEVELOPMENT OF THE LABORATORY CULTURED SEA URCHIN [1]

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The sea urchin embryo has played a key role in embryological studies for almost a century. Derbès first described the developmental process (though not with complete accuracy) in 1847. In 1876 the eggs were used in Hertwig's experiments demonstrating the role of sperm in fertilization, and in 1891, Driesch used sea urchin eggs in his experiments showing indeterminate cleavage. In recent years the sea urchin egg has become particularly prominent in the study of both the morphology and biochemistry of early development. (See reviews by Gustafson and Wolpert, 1963; Gross, 1967; Davidson, 1968.)

Probably more is now known about the early development of the sea urchin than about any other organism. The reasons for this, other than the fact that early echinoderm and vertebrate development are similar, are primarily technical. The animals are easy to obtain on almost any sea coast, they spawn readily and yield large numbers of eggs. The eggs complete meiosis in the ovaries and therefore can be fertilized immediately after spawning. Fertilization is easily accomplished simply by adding sperm to an egg suspension. Development usually follows with good synchrony and with minimum care. These eggs readily take up a large number of chemicals of biological interest, and they have the further advantage of being small and containing much less yolk than either frog or chicken eggs.

One major disadvantage in the use of sea urchins has been the extreme difficulty in raising the larvae beyond plutei to adults and thus obtaining a second generation. This has seldom been done, and never as a useable laboratory procedure. (See Harvey, 1956 for the relevant references.) If the larvae could confidently be raised, it would then be possible to apply genetic techniques to the study of sea urchin development. This, along with the other advantages these eggs offer, would make them almost ideal material to use in unraveling the developmental process.

This paper reports the first steps in that direction. It is an outline of the techniques for raising sea urchins in the laboratory, and a description of the general features of the developmental stages. The techniques have now been developed to the point where large numbers of urchins can be taken from egg to egg. Though the procedure is not yet as simple as raising *Drosophila*, it is practical.

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Life Cycle of the Sea Urchin

The life cycle of the sea urchin can be divided into six more-or-less distinct phases. (1) The fertilized egg, (2) development through blastula and gastrula to pluteus, at which time egg nutrients are usually consumed, (3) growth and development of the feeding pluteus to a mature larva, (4) development of the embryonic urchin inside the growing larva, (5) metamorphosis, and (6) growth of the young urchin to a reproductive adult. Most other Echinoderm groups develop in a similar way.

This sequence is not a smooth continuum with the structures of each stage giving rise to those of the next. The fourth one is particularly unusual. During this period the urchin, or more accurately the ventral half of the urchin, grows almost as a parasite within the larva. Most of the urchin is derived from a combination of the middle portion of the left hydrocoel and the overlying ectoderm (MacBride, 1903). Few, if any, of the larval organs give rise to comparable organs in the adult. The urchin develops its own mouth and anus, most if not all, of its internal organs, spines, *etc.* The larva comes close to being little more than a source of nutrient and protection. A similar type of embryological development is found in the insects and Nemertines. In both of these there are imaginal discs which give rise to portions of the adult. These discs also have close to an independent existence. The Echinoids differ, however, since one equivalent to a disc ultimately gives rise to the whole urchin.

The larvae

Culture Methods: The larvae of *Arbacia punctulata, Lytechinus pictus,* (Pacific Bio-Marine Supply Co., P. O. Box 536, Venice, California 90291), *Lytechinus variagatus,* (Gulf Specimen Co., Panacea, Florida 32346), *Strongylocentrotus purpuratus* and *Echinometra mathaei,* have all been raised. If several conditions are met, any of these larvae can readily be grown to maturity (*i.e.,* up to metamorphosis) in the laboratory. The proper food is most important. Larval concentration and agitation must also be controlled. Both larvae and urchins can be grown in either filtered sea water or a synthetic salt mixture such as *Instant Ocean,* (Aquarium Systems Inc., 1450 E. 289 Street, Wickliffe, Ohio), but growth has been consistently better in sea water.

The type of food organism is critical and out of 14 algal species tried, using either *Arbacia punctulata* or *Lytechinus pictus* as the test organism, only three were found to be satisfactory. These were species of *Dunaliella, Rhodemonas,* and *Pyranimonas.* All three are flagellated algae. The algae that would not serve as food were: *Amphidinium operculayum, Coccolithus huxleyi, Cryptomonas, Cyclotella nana, Cylindrotheca closterium, Eutrepticlla, Isochrysis galbana, Melosira nummuloides, Monochrysis lutheri, Nitzschia brevirostris, Phaeodactylum tricornatum.* No diatom has been found that alone could serve as food. All algae were grown in pure culture using half strength Guillard's medium (Guillard and Ryther, 1962). The larvae of *Arbacia* grew well on *Dunaliella tertiolecta. Lytechinus pictus, L. variagatus, S. purpuratus* and *E. mathaei* developed better on a diet of an alga designated 3C by Guillard and tentativelly identified as a species of *Rhodemonas. Lytechinus pictus,* and possibly the other species as well, will also develop on a species of *Pyranimonas* (designated LB 997) that was obtained from Dr. John West, University of California, Berkeley. The concentration of algae used depends on the stage of larval development, with earlier stages being fed much less than older. The larvae were usually fed once a day and given the amount of algae they would consume in 24 hrs or, in the case of young plutei, about 3000 algae per ml. No attempt was made to illuminate the larval cultures or otherwise maintain the algae. Prior to use the algae were centrifuged from their culture media and resuspended in sea water.

If mixtures of algae were used a larger number of species would probably be applicable. The natural conditions in the ocean would also be more closely approximated. However, from an experimental point of view, a single species offers a number of advantages. Maintenance of the algae is easier, feeding is simplified and any nutritional studies or radioactive labeling experiments can be better controlled.

The maximum number of larvae that can be cultured in a given volume of water depends on their stage of development. One individual per milliliter represents a comfortable maximum for mature larvae.

Some form of agitation is usually necessary during larval growth. This prevents a number of potential ills. In still water the plutei of some species tend to remain near the surface and often stick there and die. *Lytechinus pictus* is particularly susceptible to this. About half way through development there is the opposite tendency and the larvae will stay near the bottom. Here they can become trapped in debris. All this can be prevented by some form of gentle stirring. A simple apparatus for doing this is illustrated in Figure 1.

This is essentially a magnetic stirrer, but instead of placing the stirring magnets above the drive magnet, they are placed laterally. This permits a large number of stirrers to be driven simultaneously by one motor. The apparatus illustrated in Figure 1 is 20 inches high and 18 inches in diameter. The bottom shelf rests on ball bearings which allow the shelves to be rotated. Three 6 by $\frac{3}{4}$ by $\frac{1}{4}$ inch Alnico V magnets are mounted on a center shaft that is driven by a 25 RPM motor. The larvae are grown in $3\frac{3}{4}$ inch diameter by $2\frac{3}{4}$ inch polystyrene dishes (no. 42F, Tristate Plastic Molding Company, Henderson, Kentucky). Two holes approximately $\frac{3}{32}$ of an inch in diameter are drilled in the lids of the dishes, one near the edge, the other in the center. Both serve to ventilate the culture. The center hole also holds the axle of the stirrer.

An assembled stirrer is illustrated in the insert at the bottom of Figure 1. The stirrer floats inside the culture dish and consists of a small circular ferrite magnet $\frac{1}{8}$ by $\frac{3}{8}$ inches in diameter (other small magnets can work equally well) cemented inside two lids of 35 × 10 mm polystyrene petri dishes (Falcon Plastics Company, Rochester, New York). A piece of monofilament nylon fish line (approximately 20 pound test) is cemented in the top to serve as an axle. Two paddles are attached to the bottom. The particular paddle design shown, tends to minimize contact between larvae and paddle. The paddles should have a total area of about 2 cm^2 for satisfactory operation.

An alternative method is to agitate the culture by gently bubbling air through it, but this generally slows development and reduces the length of larval spines. Of the species that have been raised, *Arbacia* is the only one that grows well without agitation. In fact, it can even be raised in test tubes.

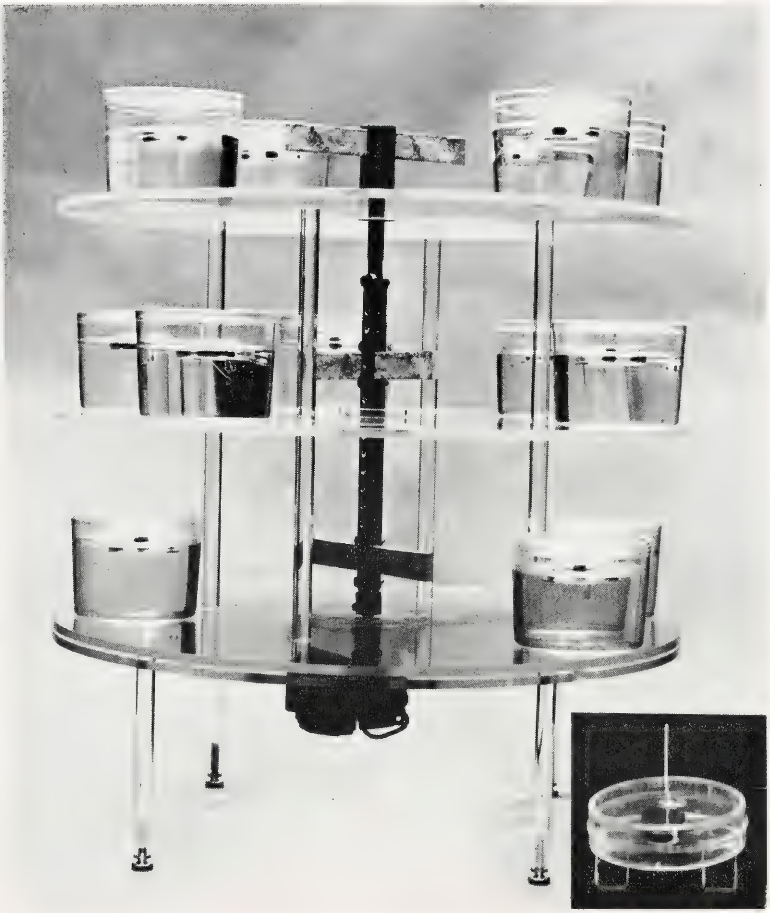

FIGURE 1. Culture apparatus, with culture dishes. Insert shows construction of the magnetic stirrer. See text for description.

The five species that have been worked with are not all equally adaptable to laboratory conditions. *Strongylocentrotus purpuratus* requires a temperature of 15° C or lower and therefore necessitates a cold room or water bath. *Arbacia, L. pictus, L. variagatus* and *E. mathaei* will all grow at room temperature (22–24° C). *Lytechinus variagatus* is the least hardy of the four. *Arbacia* is probably the easiest to raise, but the young urchins tend to hold on to any surface with tenacity and are difficult to transfer without breaking off their tube feet. The urchins are also less hardy than their larvae. The larvae of *E. mathaei* are smaller than those of the other species and this creates some difficulties, particularly in handling. The young urchin is also difficult to raise. Therefore most of what will be reported here will be results obtained with *L. pictus.* These larvae are not difficult to raise and the young urchins grow well under laboratory conditions.

Larval Development: The eggs of *L. pictus* are obtained by injection of 0.1–0.2 ml of a fresh 0.1 molar acetylcholine-sea water solution (Hinegardner, 1967).

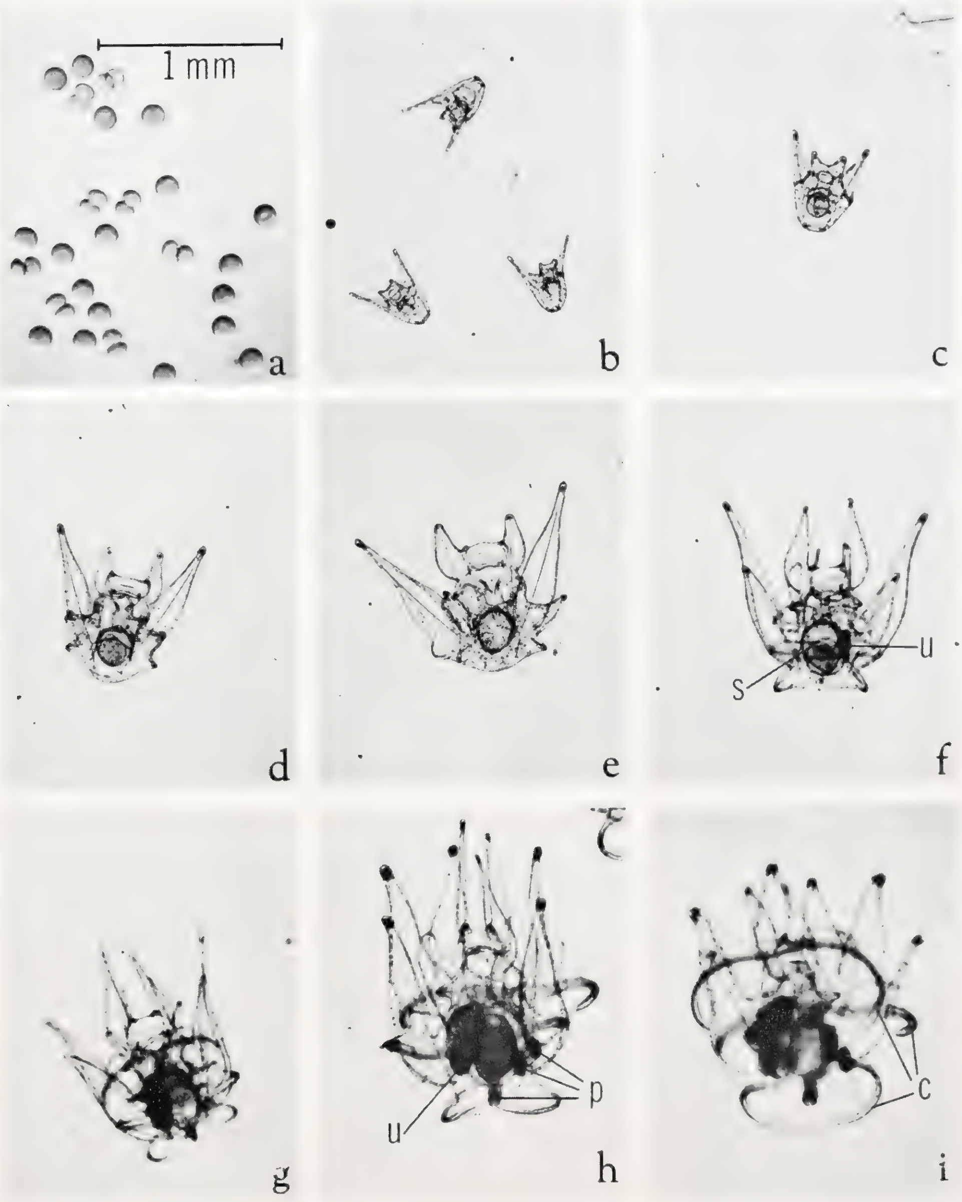

FIGURE 2. Development of the larva of *Lytechinus pictus* from fertilized egg to maturity at 24° C; (a) One and two cell embryos, (b) Pluteus, 2 days old, (c) 4 days, (d) 7 days, (e) 8 days, (f) 11 days (because of orientation the developing urchin appears on the right side), (g) 13 days, (h) 19 days, (i) 26 days. Abbreviations are: c—ciliary bands or epaulets, p—pedicillaria, s—stomach, u—urchin. All pictures are at the same magnification.

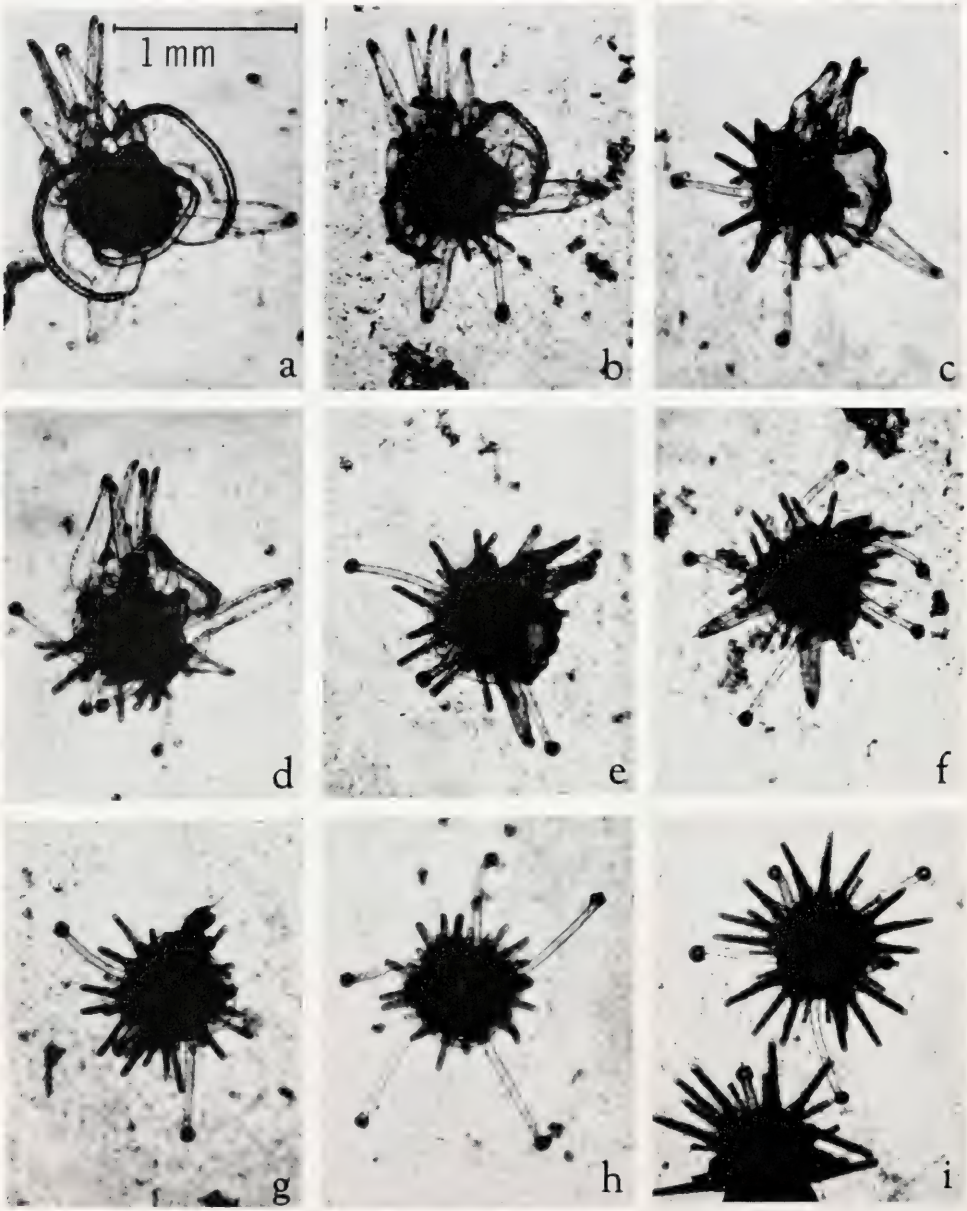

FIGURE 3. Stages in the metamorphosis of *Lytechinus pictus* larvae; (a) 1 minute after adding to the appropriate substrate, (b) 6 minutes, (c) 9 minutes, (d) 11 minutes, side view showing relation of larval structures to the emerging urchin, (e) 12 minutes, (f) 15 minutes, (g) 37 minutes, larval spines are visible in upper right, (h) 80 minutes, (i) 27 hours. All pictures are at the same magnification.

The adult urchins are more apt to survive after this treatment than after the usual 0.5 molar KCl injection or electrical stimulation. The eggs are fertilized and developed at room temperature to early pluteus in the usual way (Costello *et al.*, 1957; Tyler and Tyler, 1966; Hinegardner, 1967). In about two days the plutei are able to feed and, if conditions are close to optimum, they will reach maturity in a month or less.

Figure 2 illustrates larvae at various stages of development. The major morphological changes occurring during this period, aside from overall increase in size, are: (1) The appearance of four additional arms for a total of eight (compare Fig. 2c with Fig. 2h). (2) The formation of heavy ciliary bands which have been called epauletes, and which are most easily seen in Figure 2i. (3) The differentiation of the left hydrocoel and overlying ectoderm into the urchin tube feet primordia (Fig. 4a-c). This is followed by (4) the appearance of tooth and spine primordia and (5) urchin growth and development, primarily of the ventral half. Along with this, there is (6) the development of the three pedicellariae, one posterior and two on the right side of the larva (Fig. 2h). Finally (7) there is metamorphosis of the larva into a small urchin (Fig. 3). The development of the dorsal half and adult internal organs then begins.

Morphological details of the various stages up to metamorphosis have been described by MacBride (1903) for *Echinus esculentus.* A general description of larval development is also given in Kumé and Dan (1968).

Providing the larvae are given resonable care, about 80% of the plutei can be raised to mature larvae. It is difficult to determine what fraction of the nonsurvivors died from genetic or congential defects, but it would seem that the survival rate is close to maximum.

Metamorphosis

Loss of larval form. The physiological aspects of metamorphosis are not yet well undertood, but the visible changes that occur during this process have been followed in detail and they are illustrated in Figure 3. The first event is the settling of the larva on its left side on to an appropriate substrate. A surface covered with a mixture of algae and bacteria can induce this response. Within a minute, the anterior portion of the larva, and the arms, flex sharply toward the larva's right side (compare Fig. 3a to the normal larva in Fig. 2i). This exposes the left side of the larva and the tube feet of the young urchin to the substrate.

The tissue surrounding the urchin is then drawn up and the urchin spines appear. This is accompanied by the collapse of the larval tissue on to the top of the urchin (Fig. 3b-3g). Within about an hour (3g and h), all that remains of the larva is a lump of tissue on top of the young urchin. A few naked larval spines may extend out from it (Fig. 3g) but these are lost in a few hours. Over the next 24 hours the spines of the urchin greatly elongate (Fig. 3i).

Metamorphosis is not an obligatory stage. If the larvae are kept in clean containers they will usually not metamorphose. Instead, they continue to feed, but they grow little if at all. After two months evidence of deterioration becomes apparent and eventually they die. The maximum life span of well fed larvae is probably about four months. Their ability to metamorphose is almost com-

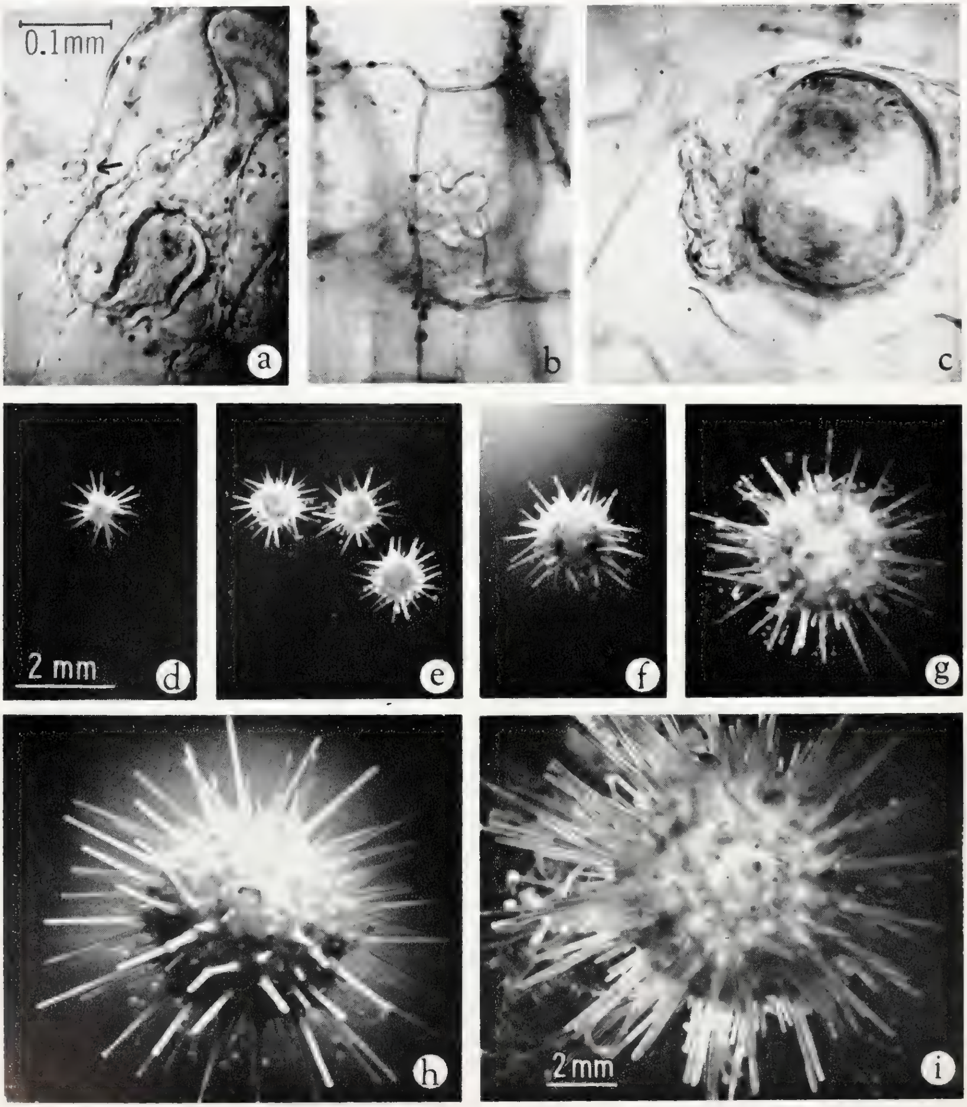

FIGURE 4. Stages in the development of the urchin; (a) 7 day old larva, arrow indicates the invaginating ectoderm; the left hydrocoel is just to the right of arrow and the gut is in the right center of the field; (b) A 9 day larva, showing the beginning of the five tube feet; this larva is rotated 90° to (a) and (c), (c) lateral view of the developing urchin in an 11 day larva; (d) 1 day old urchin; (e) 9 day urchin; (f) 27 day; (g) 71 day; (h) 117 day; (i) 208 day. (a) to (c) are taken through Nomarski optics and are at the same magnification, (d) to (h) are also at the same magnification.

pletely lost at the end of about two months. However if the larvae are underfed, this slows development and the life span can be greatly increased.

The events described here, apply to all the urchin species that have been examined. In a more general way they also apply to other echinoderm classes. Mature starfish and brittle star larvae have been obtained from plankton tows. Their metamorphosis has also been observed and it appears to follow the basic features of the pattern outlined here.

Development of the urchin. Figures 3i and 4d illustrate the young urchin. With one exception, the urchins of all the species that have been examined are very similar. However, the young *Arbacia,* which belongs to a different suborder, looks quite different (see Harvey (1956). It has 15 paddle-like spines and looks almost like a flower. The other species have 20 spines. These are cylindrical and arranged in five groups of four. In all species there are three pedicellaria which developed in the larvae and are now on the urchin's dorsal surface.

The young urchin has neither a mouth nor anus and according to MacBride (1903) no gut either. In terms of formal organs, it is little more than half an urchin; the ventral half. There is half a test, 20 ventral spines, five ventral tube feet and the five teeth. At this stage the dorsal half is essentially a rounded lump of larval tissue punctured by the three pedicellaria. The dorsal organs appear to develop out of this tissue. For the first two days the larval tissue can easily be picked off the urchin. If this is done the urchin will still continue to crawl about for a number of days afterward. The digestive system, and probably other internal organs appear at about four or five days. The urchin then begins to feed. This marks the end of the metamorphic period.

The urchin

Growth: A number of stages during the growth of *Lytechinus pictus* are shown in Figure 4. The young urchin begins to grow after it is 8–10 days old. Along with size increase, there is an increase in the number of spines and tube feet. At a shell diameter of 2 mm the madreporite begins to develop. The gonadopores appear at an age of about two months and a shell diameter of 3.2 mm. Gonads also begin to develop at this time, starting as a single lobe near the gonadopore. They grow ventrally from the gonadopores and contain some ripe gametes when the urchin reaches a diameter of 6 mm, and an age of four to five months.

Culture method: The nutritional requirements of the urchin seem to be more complex or more restricted than those of the larva and it has taken more than a year to find an appropriate algal food that could be reliably cultured in the laboratory. At present a nonsterile surface dwelling diatom is used. This has been identified as a species of *Nitzschia.* Whether or not any of the bacteria in the culture are necessary, has not been determined. Cultures of this algae are maintained in Guillard's media.

The urchins are raised in plastic petri dishes (100 or 150 mm × 25 mm). The algae is first grown in these dishes until the bottom is covered (lightly for young urchins, heavier for older urchins), then the medium is diluted 50% with sea water and the urchins introduced. The dishes are kept in an illuminated incubator at 22–24° C. Under these conditions the algae growth tends to counterbalance consumption. Conditions for a balanced ecology are hard to establish and ultimately either the algae is consumed or it grows so thick it begins to die off. In either situation the urchins are transfered to a fresh dish. If the young urchins are properly maintained they increase in diameter at a rate of approximately one millimeter in 18 days.

No real attempt has been made to determine urchin survival rates. This is because most of the urchins now being raised have been subjected to some experimental treatment. However, a rough estimate, based on experience, can be made. Survival depends, among other things, on the control of disease, maintenance of proper feeding conditions and on the particular male and female the gametes come from. Some matings appear to yield a hardier line of urchins than do others. When all the variables are taken into account, including survival of the plutei, somewhere around 50% of the young plutei can probably be grown to mature urchins. As more crosses are made within the laboratory stock, survival should improve.

Since some mature gametes can be obtained from urchins about four or five months old, a second generation can then be started. Therefore, the generation time is six months or less. Of course, no more than a few hundred eggs can be obtained at this stage, but these should be enough for genetic tests, or to establish a particular genetic line. Six months is significantly longer than the generation time of *Drosophila* but not so different from corn or mice, both of which have been used extensively in genetic studies. Therefore, if urchins are susceptible to genetic analysis, it should now be both possible and practical to use genetic techniques in the study of sea urchin development.

I wish to thank Mrs. Saundra Parra for her skilled technical assistance and Dr. R. R. L. Guillard of the Woods Hole Oceanographic Institute for kindly supplying a large number of algal species. I would also like to thank Dr. John West, University of California, Berkeley, for algae identification and for providing several algae, and Dr. Robert Kane, University of Hawaii, for sending specimens of *Echinometra.*

Summary

A method is described for raising sea urchins from egg to egg in the laboratory. The larvae are raised on flagellated marine algae and the young urchins on a substrate-dwelling diatom. The major features in the developmental process are: growth of the larva, development of the urchin inside the larva, metamorphosis and growth of the young urchin to sexual maturity. The entire life cycle takes about six months.

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ULTRASTRUCTURAL OBSERVATIONS ON THE PHAGOCYTIC UPTAKE OF FOOD MATERIALS BY THE CILIATED CELLS OF THE RHYNCHOCOELAN INTESTINE

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Previous observations at the light microscope level have shown that throughout the phylum Rhynchocoela digestion occurs by a combination of extra- and intracellular processes, with semi-digested food being taken up from the intestinal lumen for subsequent completion of digestion within intracellular vacuoles (Reisinger, 1926; Jennings, 1960, 1962; Gibson and Jennings, 1969; Jennings and Gibson, 1969). Only two cell types are present in the gastrodermis, namely pyriform gland cells whose secretions are responsible for the extracellular phase of digestion, and ciliated columnar cells in which the intracellular phase occurs. The intracellular vacuoles are relatively large (1–7 μ in diameter) and their occurrence within cells whose free distal border bears apparently normal and functional cilia poses the problem of the precise means of their formation and the method whereby materials destined for intracellular digestion enter the cells. Since the extracellular digestive phase generally reduces the food to a homogeneous semi-fluid consistency, the food vacuoles could conceivably develop from fusion of much smaller pinocytotic vesicles, which themselves formed between the bases of the cilia. That this is not the case, however, was shown by experiments in which starch grains ranging from 1–5 μ in diameter, and administered as components of test meals, appeared in the vacuoles quite unchanged and retaining their characteristic appearance when viewed by polarized light. Thus the ciliated cells can be regarded as truly phagocytic, using this term to denote intake of discrete masses large enough to be visible with the light microscope, and so far as is known this phenomenon is unique in the animal kingdom. Various other invertebrate groups possess intracellular digestion and often, as in numerous gastropod and lamellibranch molluscs, the digestive tissue also contains ciliated or flagellated components (Owen, 1966). In all these instances, though, the indications are that there is considerable structural and functional diversity amongst the cell types present, and that the ciliated cells are concerned with transporting or mixing the gut contents whilst actual uptake of particulate matter is effected by unciliated components of the gastrodermis.

In the present study, therefore, the rhynchocoelan gastrodermis has been investigated at the ultrastructural level, both in the resting condition between meals and during uptake of material from the intestinal lumen, to determine the precise means whereby particulate matter enters the columnar cells and to detect any peculiarities of the ciliated surface. Towards this last objective, comparative studies have also been made on the ciliated surfaces of the foregut, which are not concerned with digestion, and of the epidermis.

MATERIALS AND METHODS

The rhynchocoelan selected for study was the heteronemertean *Lineus ruber* (O. F. Müller), a species whose digestive physiology has been studied previously by histological and histochemical methods (Jennings, 1960, 1962; Jennings and Gibson, 1969). Specimens starved for seven days were used for studies on the resting gastrodermis; others, starved for the same length of time, were fed on clotted avian blood and fixed at intervals of 15, 30 and 60 minutes after feeding to study the entry of materials into the gastrodermal cells. Blood (from the Japanese Quail *Coturnix coturnix japonica*) was used as the test food since hemoglobin has a characteristic appearance with the electron microscope, even when partially digested, and that of an avian species was selected due to the small size of the erythrocytes which on occasion permits their phagocytosis intact.

To prevent ejection of gut contents during fixation the rhynchocoelans were first lightly narcotized n 0.36 *M* magnesium chloride. They were then transferred to fixative at 4° C and cut into pieces 1–1.5 mm in length. Fixation, dehydration and embedding in Shell Epikote resin (epon 812) were based on the procedures of Manton and Parke (1965) and Parke and Manton (1967). Initial fixation was for 2 hours in 4% glutaraldehyde in 0.1 *M* cacodylate buffer at pH 7.0 and containing 0.25 *M* sucrose, followed by three rinses, each of 20 minutes duration, in buffer containing progressively reduced amounts of sucrose (0.25 *M*, 0.125 *M* and sucrose-free, respectively) and postfixation for 1 hour in 2% osmium tetroxide in sucrose-free cacodylate. All these procedures and subsequent dehydration in graded ethanols were carried out at 4° C.

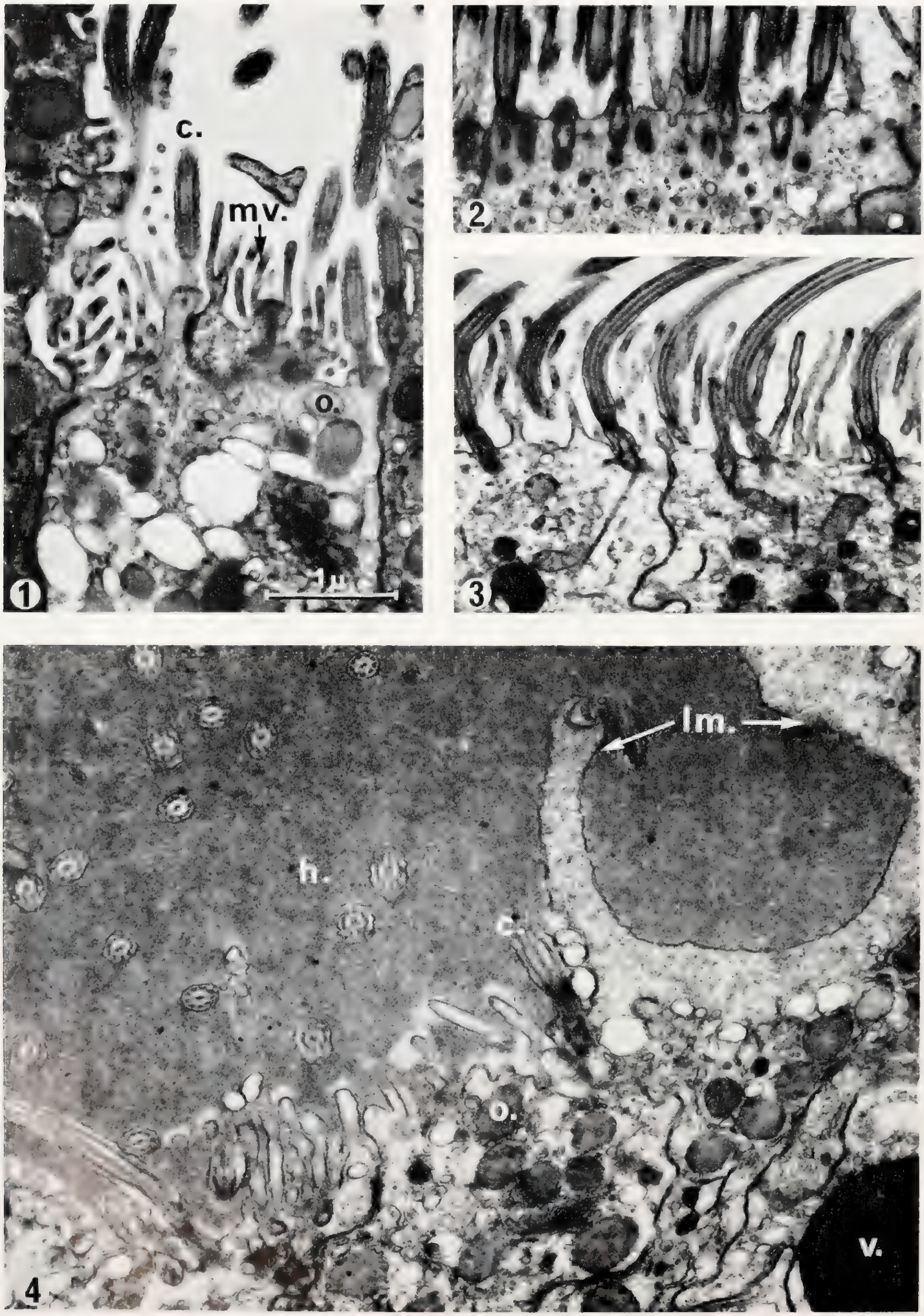
Silver to grey sections cut with glass knives on a Cambridge (Huxley) ultramicrotome and mounted on uncoated copper grids (200 mesh) were stained for 5 minutes in lead citrate (Reynolds, 1963) and examined in an AEI EM 6B electron microscope using a 50 μ or 25 μ objective aperture at 60 kv.

Other sections were cut at 0.5–0.1 μ, mounted on glass slides, stained with Azur II in borax (Jeon, 1965) and examined with the light microscope.

Attempts were made to visulaize phosphatase activity, which is a characteristic feature of the distal regions of cells concerned with food uptake in many animals, at the ultrastructural level in starved specimens by applying lead-salt replacement techniques of the type developed by Gomori (1939, 1952). For alkaline phosphatase Tranzer's (1965) method, as modified by Coleman, Evennett and Dodd (1967), was applied to glutaraldehyde fixed material which was then washed, postfixed in osmic acid, dehydrated and embedded in the standard manner. Small pieces of tissue were incubated for 1 hour at 20° C in a Gomori-type medium consisting of 0.1 ml sodium β-glycerophosphate, 6 ml 3% sodium barbitone, 1 ml 0.1 *M* lead nitrate and 1.1 ml trisodium citrate, with the final pH adjusted to 9.0 with barbiton. Inclusion of 0.1 ml 5% magnesium nitrate in the incubation medium, in pilot experiments, was found to enhance the reaction and this was eventually adopted as standard procedure.

Incubation was followed by washes in cacodylate buffer, 2% acetic acid and again in buffer, before osmication.

Acid phosphatase activity was demonstrated by a similar Gomori-type method, again based on a modification by Coleman, Evennett and Dodd (1967) of techniques described by Miller (1962) and Ericsson and Trump (1965). Incubation


1
c.
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FIGURES 1–4.

after glutaraldehyde fixation was for 1 hour at 20° C in a freshly filtered mixture consisting of 50 ml 3% sodium β-glycerophosphate and 500 ml of 0.05 M acetate buffer containing 0.6 g lead nitrate. Incubation was followed by washing in buffer and 2% acetic acid as before.

Controls for these methods consisted in each case of the incubation of comparable pieces of tissue in the standard medium devoid of the glycerophosphate substrate.

OBSERVATIONS

The distal region of the resting gastrodermis

In specimens of *Lineus ruber* starved for seven days the ciliated columnar cells of the gastrodermis are 80–100 μ tall and 6–8 μ wide. They are generally devoid of food vacuoles but possess distally a zone 10–15 μ in depth which is packed with oval bodies 0.4–1.0 μ long and 0.4–0.5 μ in diameter. These stain intensely with Azur II and at the ultrastructural level are seen to be bounded by a single membrane, often slightly crenellated, and to be moderately electron-dense after staining with lead citrate (Figs. 1, 4, and 5). A characteristic feature of the rhynchocoelan gastrodermis, as observed after application of histochemical methods, is the presence of a distal zone of alkaline phosphatase activity (Jennings, 1962; Jennings and Gibson, 1969) and in view of the similar distribution of these oval inclusions it was thought that this enzymic activity might reside in them. With the techniques employed here, however, the great majority of these bodies showed no reaction for either acid or alkaline phosphatases. Some 10–15% showed a slight reaction for alkaline phosphatase on their bounding membranes, but this did not approach the intense reaction obtained in the earlier light microscope histochemical studies in this region of the columnar cells.

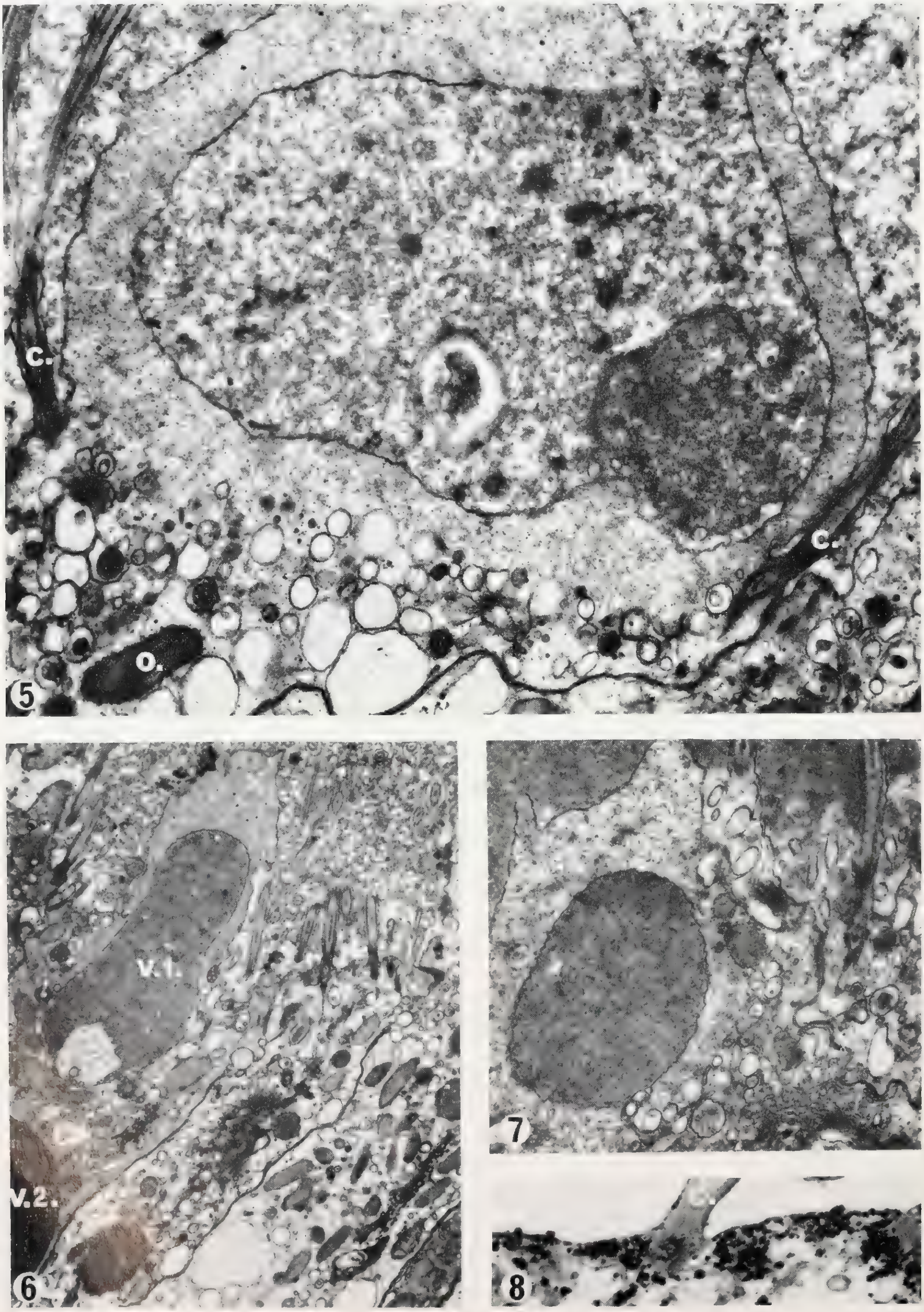
The free distal border of the cells bears flagelliform cilia, 15–18 μ long and 0.2–0.3 μ in diameter, which are distributed somewhat irregularly over the cell surface. The cilia are motile in life and ultrastructurally have the usual and characteristic internal arrangement of two central longitudinal fibers with nine other fibers arranged around them (Fig. 4). Apart from their greater length they are identical with the cilia of the foregut (Fig. 2) and epidermis (Fig. 3);

FIGURE 1. *Lineus ruber;* distal region of a ciliated columnar cell in the gastrodermis of a specimen starved for seven days before fixation; c., cilium; m.v., microvilli; o., transverse section through an oval body.

FIGURE 2. *L. ruber;* distal region of a ciliated cell in the foregut, showing cilia, basal bodies and microvilli; magnification as in Figure 1.

FIGURE 3. *L. ruber;* distal regions of two epidermal cells, showing cilia, a ciliary rootlet, microvilli and mitochondria; magnification as in Figure 1.

FIGURE 4. *L. ruber;* distal regions of three ciliated gastrodermal cells from a specimen fixed fifteen minutes after a blood meal. The intestinal lumen is filled with haemoglobin (h.) resulting from haemolysis of erythrocytes and one cell is putting out pseudopodia-like lamellae (lm.) to enclose a portion of the haemoglobin within a food vacuole. A cilium (c.) is visible adjacent to one of the lamellae and is clearly part of the same cell, whilst other cilia can be seen in transverse section amongst the gut contents. A completed vacuole (v.) with compacted and denser contents, is present deeper within the cell. The cells also show the oval bodies (o.), which are a characteristic feature of this region of the gastrodermis, and microvilli; magnification as in Figure 1.


C.
C.
O.
5
V.1.
V.2.
6
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8

Figures 5–8.

the basal bodies and cross-striated ciliary rootlets show no anomalous features, and the entire ciliary structure resembles that described in all other animal groups possessing ciliated cells.

Although no differences in structure could be found in the cilia of the gastrodermis, foregut and epidermis, there is a marked difference in their relative densities on the cell surfaces, the gastrodermal cilia being significantly fewer in number per cell as compared with the other two types and consequently much less closely packed. Thus gaps of 2–3 μ or more occur between the gastrodermal cilia throughout the length of the intestine, whereas in the foregut the cilia are extremely regular in their arrangement and occur at intervals of only 0.1–0.15 μ.

The cell surface between the gastrodermal cilia is produced into irregularly distributed microvilli, each 0.1 μ in diameter and 0.5–0.6 μ long (Fig. 1). The microvilli possess an external granular coating, or glycocalyx, but show no internal differentiation into a central core and an outer layer such as occurs in the gut microvilli of some other animal groups. They are uniform cylindrical structures and are usually orientated at right angles to the cell surface and parallel with the cilia, but sometimes they occur at varying angles and in a few instances are dichotomous, splitting into two equal sized structures 0.1 μ from the base. The microvilli are particularly noticeable in starved specimens, in which the intestinal lumen is empty.

Similar microvilli occur between the cilia of the foregut (Fig. 2) and epidermis (Fig. 3).

Uptake of food by the gastrodermis

The gastrodermal gland cells discharge their proteolytic secretions when a test meal of quail erythrocytes enters the intestine. Haemolysis, rupture of nuclear membranes and release of nuclear contents occur extremely rapidly and sections of *L. ruber* fixed fifteen minutes after feeding show the intestinal lumen to contain a granular, fairly homogeneous mass. The cilia and microvilli retain their identity and appear as in the resting gastrodermis, but the plasma membrane between these structures shows bud-like protuberances which rapidly extend outwards into the lumen contents and develop into pseudopodia-like lamellae (Figs. 4 and 5). These lamellae vary enormously in size and may eventually reach 6 μ in length. They generally arise at distances of 1–5 μ from each other and as

FIGURE 5. *L. ruber;* distal region of a ciliated gastrodermal cell thirty minutes after a blood meal, showing two lamellae which have almost completed formation of a food vacuole. The lamellae are each flanked by a single cilium (c). o., oval body seen in longitudinal section; magnification as in Figure 1.

FIGURE 6. *L. ruber;* distal portions of three ciliated gastrodermal cells, thirty minutes after feeding. The cell to the left shows a recently completed vacuole (v.1.), flanked by a cilium, and below this is an earlier vacuole (v.2.) with much denser contents. The other cells show cilia and microvilli; magnification one half of Figure 1.

FIGURE 7. *L. ruber.* Portion of the distal region of a ciliated gastrodermal cell, fifteen minutes after feeding, showing a recently completed vacuole. Magnification as in Figure 1.

FIGURE 8. *L. ruber.* Extreme distal region of a ciliated gastrodermal cell in a specimen starved for seven days. The black granules represent sites of alkaline phosphatase activity. c., cilium. Tranzer's method, as modified by Coleman, Evennett and Dodd. Magnification as in Figure 1.

they extend outwards into the gut lumen tend to curve downwards somewhat, back towards the cell surface. Eventually the tips of two lamellae meet and fuse, trapping a mass of semidigested food between themselves and the cell surface (Figs. 6 and 7). In this way a vacuole up to 5 or 6 μ in diameter, containing semidigested food and bounded by a single membrane, is formed and this passes back into the cell for subsequent intracellular digestive processes. As the newly formed vacuole moves away from the cell surface more lamellae develop and the entire process is repeated several times. Thus within thirty minutes of feeding most of the ciliated gastrodermal cells show two or more vacuoles in their distal regions (Figs. 4 and 6). The contents of the vacuoles become compacted and denser in appearance as they pass deeper into the cells, presumably as water is absorbed and digestion begins.

Throughout this process the gastrodermal cilia remain distinct, and clearly are not concerned in any way with the uptake of materials from the lumen. In many instances the origin of a lamella lies extremely close to a cilium (Figs. 4 and 5), but it is always distinct from any part of the ciliary apparatus. The microvilli are often less distinct during the phagocytic process and were in fact absent from many fields examined, especially in sections of rhynchocoelans fixed thirty or sixty minutes after a meal. When clearly visible they showed no significant differences in structure or disposition as compared with their appearance in the resting gastrodermis.

No evidence was found to suggest that the food vacuoles ever form by invagination and vesiculation of the plasma membrane and it would appear, therefore, that the entry of visible food materials into the ciliated columnar cells is the result of a normal phagocytic process, with pseudopodia-like lamellae protruding from the cell surface between the cilia to engulf the food and enclose it within a typical food vacuole.

Phosphatases in the ciliated cells

Throughout the gastrodermis a positive reaction for alkaline phosphatase was obtained in the extreme distal region of the ciliated columnar cells, the reaction being concentrated in a narrow band 0.1–0.2 μ in depth immediately below the plasma membrane (Fig. 8). A much more scattered and diffuse reaction was obtained in the cells to a depth of 20–30 μ from the distal region. In neither case was the reaction associated with any organelle, apart from the slight reaction mentioned earlier on the limiting membrane of a small proportion of the oval bodies found in this region of the cell. Only starved *L. ruber* were used in this part of the study, and no observations were made on the occurrence or distribution of alkaline phosphatase during phagocytosis.

The only reaction obtained for acid phosphatase was in lysosomes occuring in the basal portions of the ciliated cells in the region of the nuclei.

DISCUSSION

The evidence presented here shows that the food vacuoles of the rhynchocoelan gastrodermis result from a truly phagocytic process, in which temporary outgrowths of the cell surface meet to enclose material lying in the gut lumen. The vacuole formed in this way then passes back into the gastrodermal cell for subsequent intracellular digestion of its contents. This phagocytic process occurs between the cilia of the gastrodermal cells and is quite independent of them. The only modification apparent in the gastrodermal cilia which can be construed as related to phagocytosis is their relatively sparse distribution over the cell surface, when compared with the more densely packed cilia of the foregut and epidermis. This, presumably, is to leave adequate surface area available for development of the phagocytic outgrowths at the appropriate stage of the digestive process. The greater length of the gastrodermal cilia, relative to those of either the foregut or epidermis, may well in turn be an adaptive feature which compensates for this reduction in the number of cilia present per unit area of cell surface.

An earlier interpretation of this phenomenon of phagocytosis by ciliated cells, based entirely on observations with the light microscope, was that the cilia themselves somehow coalesced into pseudopodia-like structures which engulfed material from the gut lumen (Jennings, 1960). A re-examination of the material used in the earlier study, in the light of the present work, shows that the apparent coalescence of the cilia at the time when food vacuoles are appearing in the gastrodermis is a fixation artefact, adjacent cilia and cell outgrowths having clumped together into structures whose precise nature cannot be resolved by the light microscope.

The microvilli which occur between the cilia of the gastrodermis, foregut and epidermis are a fairly common feature of ciliated epithelia and have been described in a variety of tissues from both invertebrate and chordate groups (Fawcett and Porter, 1954; Porter and Bonneville, 1964; Iwai, 1967a, 1967b). The gastrodermal microvilli in the rhynchocoelan are probably concerned with absorption of simple soluble materials which are either present initially in the food or produced during the first, extracellular, phase of digestion. Ugolev (1960, 1965) has presented sound evidence for the participation of the microvilli of the vertebrate intestine in the actual digestive process, as contact catalysts ensuring intimate mixing of enzymes and substrates, and there are indications that they play the same role in some invertebrates such as digenetic trematodes (Halton, 1966) and nematodes (Colam, 1969). It seems unlikely, however, that the microvilli of the rhynchocoelan gastrodermis can be at all effective in this way, since, like the gastrodermal cilia, they are sparsely distributed over the cell surface and do not occur as the tightly packed and regularly arranged outgrowths seen in other animal groups.

The role of the foregut microvilli remains obscure. There is no digestion in this part of the gut in any rhynchocoelan so far studied and food does not remain in it for more than a few seconds (Jennings and Gibson, 1966). It is difficult to see, therefore, any possible role for these microvilli unless there is absorption here of excess water taken in through the mouth with the food.

The microvilli of the rhynchoelan epidermis were first described by Fisher and Cramer (1967) in *Lineus ruber*. These authors also demonstrated absorption of glucose and amino acids across the epidermis and concluded that the microvilli are involved in this process. Jennings and Gibson (1969) reported the presence of non-specific esterase activity in the epidermis of *L. ruber* and suggested that the enzyme responsible may be concerned with extra-corporeal digestion of simple proteins or polypeptides, the products then being absorbed by the mechanism described by Fisher and Cramer. In this connection it is interesting to note that Jennings and Gibson also found non-specific esterase activity in the foregut of *L. ruber,* at sites and in quantities comparable to those in the epidermis. Thus the same sort of link may exist in the foregut, between microvilli, non-specific esterase activity and absorption as is suggested for the epidermis.

The alkaline phosphatase activity found in the distal regions of the columnar cells and the acid phosphatase localized in lysosomes presumably correspond to the enzymic activities visualized in the cells in earlier, histochemical, studies (Jennings, 1962; Jennings and Gibson, 1969). Their precise roles in the intracellular digestive processes are at present under investigation at the ultrastructural level and will be reported in a later account.

SUMMARY

1. Ultrastructural observations on the rhynchocoelan *Lineus ruber* confirm that the ciliated columnar cells of the gastrodermis are truly phagocytic, in that they engulf visible particulate material from the intestinal lumen, enclose it in food vacuoles and pass these back into the cell for subsequent intracellular digestion.

2. The cilia have the form and structure that is ubiquitous for these organelles throughout the animal kingdom and they are not concerned in any way with the phagocytic uptake of material from the gut lumen.

3. After the initial extracellular digestion, which renders the food semi-fluid and relatively homogeneous, the cell surfaces between the cilia develop pseudopodia-like outgrowths or lamellae which extend out into the gut lumen. The tips of two adjacent lamellae fuse to form a vacuole enclosing semi-digested food and the vacuole then moves back into the cell for completion of digestion.

4. Lamellae are only present when semi-digested food is present in the intestine and they are absent from the resting gastrodermis between meals.

5. Microvilli occur between the gastrodermal cilia and are believed to be concerned with absorption of simple soluble substances from the gut lumen. They also occur between the cilia of the epidermis and of the foregut.

6. A zone of alkaline phosphatase activity, not associated with any particular organelle, occurs in the extreme distal region of the columnar cells. Acid phosphatase activity can be demonstrated in lysosomes occurring in the basal regions of the cells.

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TEMPERATURE ADAPTATIONS OF COPEPOD EGGS FROM THE ARCTIC TO THE TROPICS

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The purpose of this paper is not simply to show once again that temperature adaptation occurs in physiological processes of poikilotherms from different latitudes. It also demonstrates what appears to be the simplest way of expressing such adaptation by a single parameter, and suggests that the relationship between this parameter and environmental temperature may be sufficiently close to allow prediction of development rate of copepod eggs from different latitudes.

Use is made of an expression developed by Bělehrádek (1935) relating physiological rates and temperatures:

$$V = a\,(T - \alpha)^b$$

where V is the observed rate, T is the temperature, and *a, b* and α are fitted constants. Other three-constant equations may be equally accurate, but, as has been shown elsewhere (McLaren, 1963), this equation reveals differences between rate-temperature curves in the simplest and most direct way. Each constant defines a unique property of such curves: *a* describes differences in slope, *b* is adequate to account for differences in monotonic curvilinearity within *natural* temperature ranges and α ("biological zero") may be well below the natural temperature range, but defines positions of curves on the (arbitrary) Celsius scale.

It has long been known that large eggs tend to develop more slowly than small eggs among related organisms (Berrill, 1935). For different races of the frog *Rana pipiens* in the United States, choice of a single estimate of *b* for all races reveals a simple proportionality between egg diameter and *a* of Bělehrádek's equation (McLaren, 1965). The same is true among different populations of the same species or among closely related species of copepods (McLaren, 1966). Another equation, actually isomorphic with Bělehrádek's with *b* taken as −1.0, has been used to describe development time of nematode eggs (Crofton and Whitlock, 1965); the β of their equation, equivalent to *a* in Bělehrádek's, was shown to be close related to egg volume. There is, therefore, empirical justification for assuming that a single value of *b* applies among related species, since doing so reveals a simple relationship between egg size and development rate.

If *b* is the same among related species, and *a* is a constant of proportionality, then α remains as the "real" indicator of temperature adaptation. A previous

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TABLE I

Development times to hatching of copepod eggs at different temperatures. Each experiment is a single egg sac carried by a female, or a batch of eggs produced more-or-less synchronously by one or more females

Species	Locality	Number of experiments	Mean temperature (°C)	Days to hatching	
				Mean	Range
Pseudocalanus minutus	Halifax	17	0	10.70	10.07–11.14
		11	2.73	6.92	7.71– 7.16
		4	5.35	5.06	4.90– 5.31
		11	9.27	3.48	3.38– 3.58
		8	11.43	3.06	2.79– 3.56
		14	12.01	2.90	2.75– 3.00
Acartia clausi	Halifax	2	0	15.58	15.33–15.84
		3	4.88	5.98	5.88– 6.05
		3	10.80	2.78	2.69– 2.87
Tortanus discaudatus	Halifax	1	0	19.71	
		3	4.85	8.15	8.00– 8.25
		3	10.80	3.93	3.82– 3.98
Calanus glacialis	Frobisher, N.W.T.	1	0	6.35	
		1	2.60	4.30	
		1	5.23	3.23	
		1	7.92	2.61	
Calanus hyperboreas	Halifax	3	0	6.67	6.50– 6.75
		2	3.34	4.30	4.23– 4.38
		4	6.89	2.98	2.90– 3.02
Metridia longa	Halifax	2	0	4.20	
		2	3.08	2.93	2.88– 2.98
		1	3.53	2.76	
		2	7.69	1.73	
		1	11.96	1.34	
Temora longicornis	Halifax	2	0	10.81	10.79–10.84
		3	3.26	6.47	6.35– 6.55
		3	7.39	3.71	3.52– 3.83
		6	11.83	2.32	2.27– 2.38
Eurytemora hirundoides	Halifax	1	0	13.27	
		2	3.17	7.80	7.74– 7.85
		1	11.80	2.82	
Centropages furcatus	Jamaica	1	15.85	2.83	
		3	17.60	2.00	1.96– 2.08
		3	20.21	1.17	1.08– 1.27
		3	25.78	0.74	0.66– 0.86
		3	29.43	0.58	0.50– 0.66
Acartia tonsa	Narragansett Bay	1	10.0	6.0	
		38	11–12	4.75	4.17– 5.33
		6	14–15	3.0	2.80– 3.20
		5	15.4–16.2	2.3	2.01– 2.59
		5	24.5–26.1	0.68	0.66– 0.70
Calanus finmarchicus	Tromso, Norway	6	0		4.83– 5.41
		12	5		2.46– 2.71
		9	13–14		0.96– 1.08
		3	20		0.83– 1.00

study (McLaren, 1966), largely concerned with the effects of egg size and yolk concentration on the value of *a*, revealed only slight differences in α for egg development among several geographically distant populations of the copepod *Pseudocalanus minutus*. However, α was 3–4° C lower for three species whose ranges extend to arctic waters than for two temperate species. The present paper extends the analysis to several more species, and attempts a more direct determination of the relationship between α and environmental temperature.

MATERIALS AND METHODS

Experimental methods have been described elsewhere (McLaren, 1966). Briefly, each experiment on Table I involved a single egg sac carried by a female or a group of free eggs produced more-or-less synchronously by one or more females, and kept at controlled temperatures until all viable eggs hatched. Experimental salinities were not always determined, but the influence of salinity on development rate is probably negligible within natural ranges (McLaren, Walker, and Corkett, 1968).

RESULTS

Development times (with fiducial limits) of the first four species on Table I have been published previously (McLaren, 1966), but additional experiments have been added for two of these.. Data for *Calanus finmarchicus* are from Marshall and

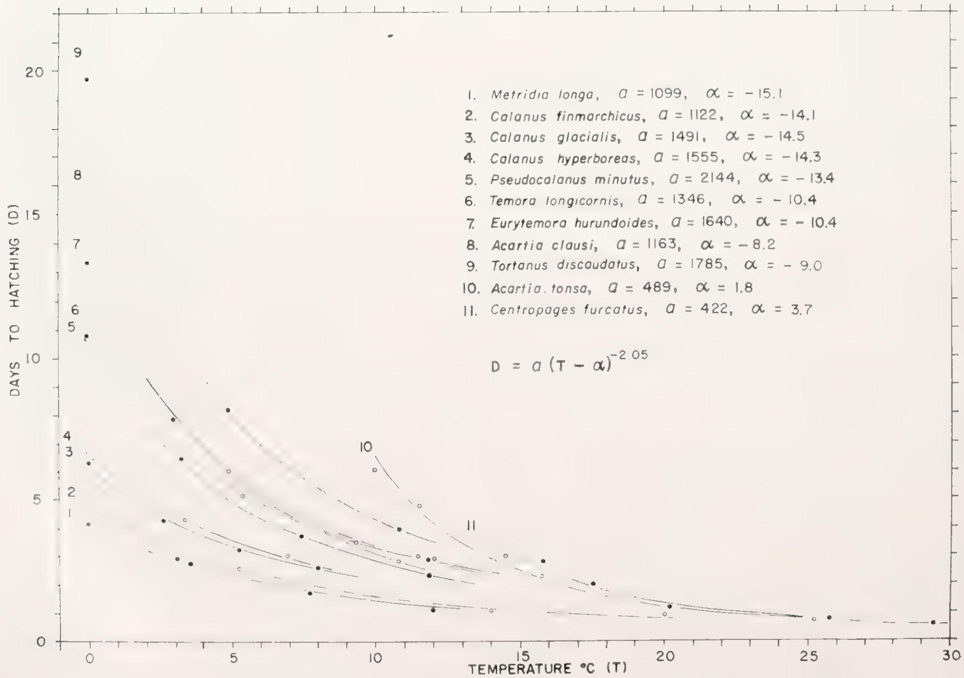

FIGURE 1. Bělehrádek's temperature functions fitted to mean or median times to hatching of copepod eggs (data on Table I).

Orr (1953). Resting eggs were reported earlier in *Acartia tonsa* (Zillioux, 1969) and some eggs in this study also showed dormancy at 14–15° C and below. The values on Table I are, however, for normal, uninterrupted development.

Bělehrádek's temperature functions were fitted to determine the best single estimate of b for all species on Table I except *Acartia tonsa* and *Calanus finmarchicus,* for which temperatures and times are available only as ranges in some instances. This was done by successive approximation by computer to the value of b (nearest 0.05) giving the smallest sum of standard errors of the regressions for all points for each species: $\log$ (development time) $= \log a + b \log (-\alpha)$. This value of $b = -2.05$ was then used to estimate a and α from unweighted median times and temperatures for *A. tonsa* and *C. finmarchicus* as well.

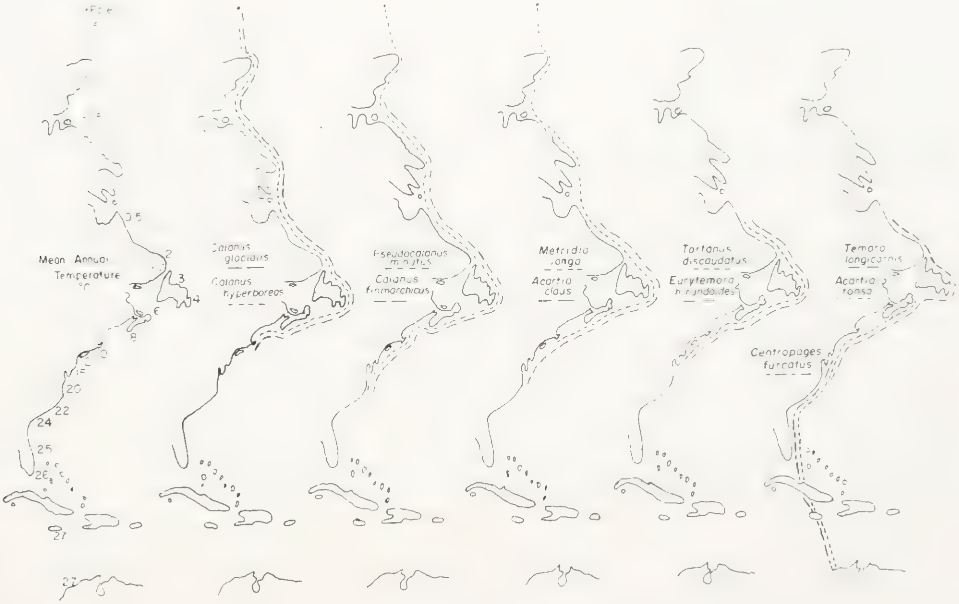
Pole
Mean Annual Temperature °C
0.5
2
3
4
6
8
10
15
20
22
24
25
26
27
Calanus glacialis
Calanus hyperboreos
Pseudocalanus minutus
Calanus finmarchicus
Metridia longa
Acartia clausi
Tortanus discaudatus
Eurytemora hirundoides
Temora longicornis
Acartia tonsa
Centropages furcatus

FIGURE 2. Mean annual temperatures and distributions of copepods between the North Pole and South America.

The resulting curves, transformed to a linear plot, are shown with their respective constants on Figure 1. Clearly the curves fit the various data well (the correlation coefficients of the logarithmic regressions exceed 0.99 for all species except *Centropages furcatus* at 0.98). The deviations of points for *A. tonsa* may be attributed to use of medians for times and temperatures (*cf.* Table 1).

A proportionate relationship between a (Fig. 1) and egg diameter is confirmed for the three species of *Calanus.* Eggs of C. finmarchicus are about 145 μ in diameter (Marshall and Orr, 1953), those of *C. glacialis* are about 179 μ (McLaren, 1966) and a single measured egg of *C. hyperboreas* was 190 μ. However, an earlier conclusion (McLaren, 1966) based on limited data, that greater yolkiness of eggs would slow down their development rate and therefore increase a, is not born out. Eggs of *C. hyperboreas* are much more opaque, apparently because large amounts of yolk stored for non-feeding young stages (Conover, 1967), than are eggs of *C. glacialis* (McLaren, 1966), but *a* values of these species seem to be explicable in terms of egg size alone.

The values of α on Figure 1 show a logical relationship with environment; northern forms have low values and warm-water species have high values of α. Much information on geographical distributions of the species on the east coast of the United States is summarized by Deevey (1960) and Wilson (1932). Northern limits can be established for northern Labrador (Carter, 1965), Ungava Bay (Fontaine, 1955) and the high arctic of Canada and the Arctic Ocean (Grainger, 1965). Range maps are available for the three species of *Calanus* in North America (Grainger, 1963). *Eurytemora hirundoides* is recorded from as far south as Chesapeake Bay (Wilson, 1932) and as far north as Northern Labrador (Carter, 1965); some or all records may be referred to *E. affinis* (Deevey, 1960), but this probably does not affect the results in this paper. Distributions of each species on Table I between the North Pole and South America are summarized on Figure 2. The animals may, of course, be seasonal or local within these ranges, and doubtless successful breeding is geographically more restricted.

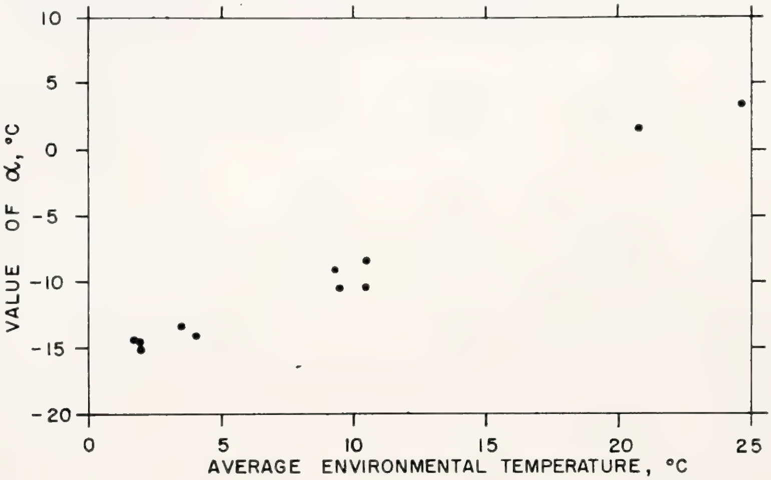

FIGURE 3. Relationship between α ("biological zero") of Bělehrádek's temperature function for eggs of eleven species of copepods and estimates of average temperature in their environmental ranges.

Among geographically widely separate populations of *Pseudocalanus minutus* and *Calanus finmarchicus*, α varied less than 1° C (McLaren, 1966). This suggests that α is genetically adapted to conditions of the species' whole geographical range (and allows the use of α as determined from experiments on *Calanus finmarchicus* from Norway to approximate the value of α for the same species in North America). There is no precise way to determine the average temperatures to which a species may be subjected, but a crude approximation has been attempted by using mean annual surface temperatures calculated from mean monthly surface temperatures

(Meteorological Office, London, 1948; U. S. Navy Hydrographic Office, 1957) along the latitudinal ranges of the species (Fig. 2). The resulting relationship between α and "average environmental temperature" is shown on Figure 3.

DISCUSSION

We conclude that a single parameter of temperature response (α) can be used to describe differences in temperature adaptation of development rate of eggs of related species. The relationship between α and approximate environmental temperature is so close that it may be possible to predict development rate at any temperature of eggs of copepods of known geographical range from experiments at a single temperature. It should be understood that these approaches do not depend on any theoretical assumptions. The mathematical resolution of such three-constant functions is rather low so that purely empirical curves, in which all constants are fitted, may show wide variations in the values of b. Assuming that b has the same value for all species may have no biological basis, and may not even be correct within specified limits of accuracy. The justifications for this assumption are the statistical adequacy of the resulting curves (Fig. 1), the aforementioned relationship between egg size and a, and the equally striking relationship between α and evironmental temperature that is revealed in this paper. Clearly these relationships are of some interest.

The results may also be compared with certain traditional approaches to temperature adaptation. The approach seems much simpler than attempting to quantify relationships between environmental temperature and differences in "Q_{10}," shifts in weight-metabolism curves, "rotation" or "translation" of semilogarithmic plots, and other similar modes of expression (examples in Rose, 1967). A recent analysis of temperature adaptation of amphibian embryos by Bachman (1969), who does not refer to an earlier analysis of some of the same material (McLaren, 1966), assumes that the relationship between temperature and development rate is linear within the temperature range that permits high survival of embryos. This is equivalent to using Bělehrádek's equation with $b = 1.0$, and leads to values of "$T_0 + 10$" ($= \alpha + 10$) as a measure of temperature adaptation. (In using development time at $T_0 + 10$ as another measure of temperature adaptation, Bachman fails to note the independent effects of egg size described earlier by McLaren.) Determining $T_0 + 10$ from a linear regression may be arithmetically simpler, but cannot be generally accurate; development rate of copepod eggs, for example, is more nearly a square function ($b = 2$) than a linear function of temperature.

Although the usefulness of Bělehrádek's function does not depend on theoretical assumptions, it is tempting to attach some biological meaning to its constants. The ways by which egg size might affect the value of a have been discussed in previous papers (McLaren, 1965, 1966). Although differences in a may in some sense be involved in temperature adaptation—since rates will be speeded up or slowed down at any temperature—it seems intuitively satisfying to think of true temperature adaptation simply as a shift of the entire curve on the temperature scale as expressed through changes in α. Whether the underlying mechanisms involve changes in biophysical restrictions on biochemical rates (Bělehrádek, 1935; McLaren, 1965, 1966) or are explicable by more sophisticated reaction-rate theories (*e.g.,* Hochachka and Somero, 1968) it can be argued that molecular explanations of adaptation in development rate of copepod and other eggs should account for the rather simple relationship between α and environmental temperature.

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SUMMARY

The development times to hatching (D) of eggs of eleven species of copepods are closely described as functions of temperature (T) by Bělehrádek's temperature function, $D = a\ (T - \alpha)^b$ using the assumption that the same value of b applies to all species. The value of a is related to egg diameter among three species of *Calanus,* and is unaffected by the greater opacity (presumably yolkiness) of eggs of *C. hyperboreas.* The value of α remains as the "real" indicator of temperature adaptation, and is closely and linearly related to estimates of environmental temperature based on mean annual temperatures within the range of each species between the pole and South America, along the east coast of the Americas.

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HYPERBARIC OXYGEN AND SUCCINIC DEHYDROGENASE IN TUBULARIAN DEVELOPMENT[1]

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In 1938, Barth reported that regeneration of *Tubularia* required oxygen and that the size of the regenerating hydranth was directly related to the volume of the gas dissolved in the ambient seawater. His studies embraced the range from 0 ml O_2 per liter to 21.6 ml O_2 per liter. The latter represents the equivalent of 1 atmosphere of pressure and produced the highest rates of regeneration. There have been no studies upon the effects of higher concentrations of oxygen produced by hyperbaroxia either upon regeneration in *Tubularia* or on early development of this hydroid, although his data did not show that the maximal effects of oxygen had been reached. Studies in our laboratory show that 2 to 4 atmospheres absolute of oxygen block differentiation but not the cell migration which occurs during regeneration in this species (Miller, DeSha, Heidger and Miller, 1966).

A striking correlation has been found in *Tubularia* between the early differentiation of organs (tentacles, gonophore buds, perisarc-secreting zone, *etc.*) and the localized increase in succinic dehydrogenase activity (Miller, Hegab and Miller, 1964; Miller *et al.*, 1966). Since Stadie and Haugaard (1945) found that hyperbaric oxygen blocks succinic dehydrogenase activity, a study of the effect of hyperbaroxia is of particular interest in this species in which the enzyme is known to be so closely related to developmental processes.

MATERIAL AND METHODS

Colonies of *Tubularia* sp. were collected from the Cape Cod Canal, transported in iced containers and placed in beakers of running, aerated seawater at 16° C. Hydranths of gravid female colonies were removed and placed in large fingerbowls filled to a depth of 1 ml with filtered seawater (Miller, 1959) approximately 50 hydranths per fingerbowl. During the subsequent 2 to 6 hours at room temperature the gonophores shed their young, first the actinulae and later, younger and younger stages until finally uncleaved eggs were released. Harvesting was done frequently and the young were placed in large volumes of fresh filtered seawater and kept at 15–16° C until the beginning of the experiment. After selection of the stage to be used, the embryos were placed in Petri dishes containing approximately 30 ml of filtered seawater in a cold room which was maintained at 15° C ± 1°. The experimental animals were exposed to gas at the desired pres-

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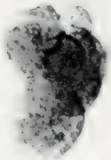

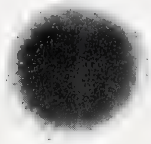

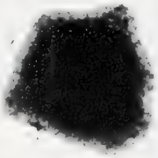

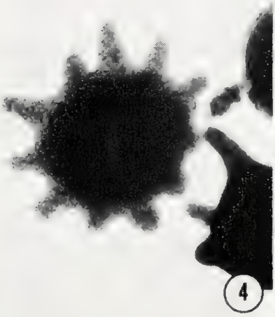

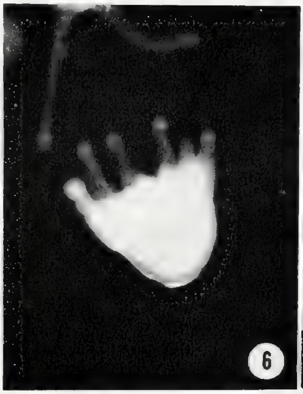

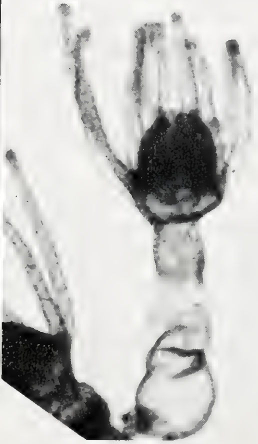

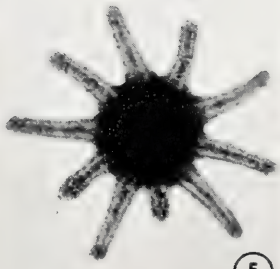

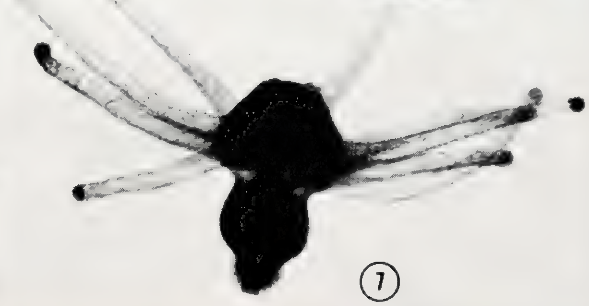

Figure 1. Cleavage: this shows the chaotic nature of the cell divisions.

Figure 2. Morula: an apparently undifferentiated ball of cells.

Figure 3. Polygon: the points on the disk indicate location of future proximal tentacles.

Figure 4. Early Star: the tentacles are short.

Figure 5. Late Star: the tentacles are elongated but no aboral projection is visible.

Figure 6. Basket: growth of the aboral surface has caused the tentacles to project more distally. Dark field photograph.

Figure 7. Actinula: the distal tentacles are developing. The aboral surface has differentiated the holdfast (the most distal knob) and the perisarc-secreting growth zone (the subterminal thickened ring).

Figure 8. Polyp: the aboral end has attached to the substrate and the growth zone has produced a short stem.

sure in a Bethlehem Co. Table-Top Hyperbaric Chamber. Observations were made and the stages of the embryos were recorded at approximately 24 hour intervals.

At the time of the observations the Petri dishes were refilled with fresh filtered seawater in order to reduce the numbers of bacteria and protozoa. Because deaths in controls increased during the latter part of the season at a time when colonies were heavily infested with protozoa (*cf.* Table IV) the subsequent experiments were performed using pasteurized seawater (20 min at 70° C).

Succinic dehydrogenase activity was assessed by a modification of the method of Nachlas, Tsou, DeSouza, Chang and Seligman (1957) which was used in our previous study (Miller *et al.*, 1964; Miller *et al.*, 1966).

The following stages in the development of *Tubularia* were used in recording observations (Figs. 1–8):

1. Uncleaved egg.

2. Cleavage stages. These are so irregular as to be characterized as chaotic. Cleavage usually begins at the pointed end of the egg and progresses along the external (future aboral) surface more rapidly than along the internal (Fig. 1).

3. Morula, a multicellular ball (Fig. 2).

4. Saucer, a flattened ball wrapped around the gonostyle.

5. Polygon. This is a brief period when the proximal tentacles are first beginning to form. It is named because of its characteristic shape and because the endoderm (but not the ectoderm) of the tentacles shows great activation of succinic dehydrogenase at this stage (Fig. 3).

6. Star. In this stage the proximal tentacles are less than 4 or 5 times as long as wide and there has been an increase in succinic dehydrogenase activity in the ectoderm at the base of the tentacles (Figs. 4 and 5).

7. Basket. Stars transform into baskets as a result of the growth of the aboral surface. As a result the tentacles change from a laterad to distad orientation (Fig. 6).

8. Early Actinula. The growth of the hypostome region and the thickening of the cells which form the perisarc-secreting zone and the adhesive organ give the early actinulae their characteristic appearance.

9. Late Actinula. Late actinulae are distinguished from early stages by their more elongated shape which results both from continued growth of the hypostome region and the perisarc-secreting zone and by the presence of distal tentacles (Fig. 7).

10. Polyp. With the attachment of the actinula to the substrate, the secretion of perisarc and growth of posthydranth tissues the larval period is terminated and its life as a sessile organism commences. Accordingly this has been designated the polyp stage (Fig. 8).

RESULTS

Table I summarizes data on survival of controls and of oxygenated embryos. It shows that exposures to oxygen at hyperbaric pressures between $1\frac{1}{2}$ and 4 atmospheres absolute protected embryos from death during 48 hours of exposure. Although because of their small size, some embryos may have been lost in changing water, care was taken to keep attrition by this route at a minimum and therefore

TABLE I

48 hour survival of control and oxygenated embryos

	Morula	Star and basket	Actinula	Total
Control	63.7%	73.9%	92.9%	73.0%
Oxygen				
$1\frac{1}{2}$ AA	80.0%	80.0%	100%	86.7%
$2–2\frac{1}{2}$ AA	80.3%	—	—	80.3%
3 AA	77.0%	86.3%	—	83.8%
4 AA	84.0%	95.0%	96.0%	92.5%
Totals:				
Control	63.7%	73.9%	92.9%	73.0%
surv./total	(109/171)	(159/215)	(65/70)	(333/456)
Oxygen	80.7%	89.8%	97.1%	87.5%
surv./total	(138/171)	(193/215)	(68/70)	(399/456)

it can be assumed that very few embryos were lost, and that these losses were approximately equal in control and experimental groups. In addition, fragments of embryos were commonly seen in the control containers but were rare in the oxygenated lots. As a consequence it was concluded that the differences in numbers between the hyperbaric and control animals were real and represented differences in death rates in the two groups. It is to be noted that survivals were lowest and protection by oxygen greatest in the early embryos. When pasteurization was used in addition to filtration survivals were highest in both oxygenated and controls (Tables IV and V). If survival is used as a criterion it may be concluded that hyperbaric oxygen is beneficial within the range tested ($1\frac{1}{2}$–4 atmospheres).

TABLE II

Efects of oxygen at $1\frac{1}{2}$ atmospheres absolute on development of tubularian embryos

Before expt.		Treatment	At 48 hours		
Stage	No.		Actinulae		Dead or lost
			Early	Late and polyp	
Morula	20	Control	0	11	9
	20	$O_2–1\frac{1}{2}$ AA	0	16	4
Star and Basket	20	Control	0	15	5
	20	$O_2–1\frac{1}{2}$ AA	0	16	4
Actinula	20	Control	5	15	0
	20	$O_2–1\frac{1}{2}$ AA	0	20	0
Totals	60	Control	5	41	14
	60	Oxygen	0	52	8

TABLE III

Effects of oxygen at 2 and $2\frac{1}{2}$ atmospheres on development of tubularian morulae

Treatment	No. of morulae	At 48 ± hours						
		Morula	Polygon	Basket	Actinula		Polyp	Dead or lost
					Early	Late		
Control	28	4	1	0	4	*8*	4	7
O_2–2 AA	28	4	1	*9*	4	0	0	10
Control*	38	3	1	0	5	*15*	0	14
O_2–$2\frac{1}{2}$ AA*	38	9	3	*16*	5	0	0	5
Totals								
Control	66	7	2	0	9	*23*	4	21
O_2–$2+2\frac{1}{2}$	66	13	4	*25*	9	0	0	15

* Two lots.

Table II summarizes the effects of oxygen at $1\frac{1}{2}$ atmospheres on the development of embryos and larvae of three different stages: (1) morula, (2) star and basket and (3) actinula. The data suggest that morulae but not later stages may be affected by $1\frac{1}{2}$ atmospheres of oxygen.

Table III shows that 2 or $2\frac{1}{2}$ atmospheres of oxygen are definitely inhibitory. None of the morulae subjected to 2 atmospheres reached late actinula stage whereas 8 controls reached this stage and 4 attached to the substrate and became polyps. In this and subsequent tables the italics indicate the largest group. With $2\frac{1}{2}$ atmospheres of oxygen, development was slowed greatly and also appeared to be blocked at the early actinula stage, with the majority of the embryos in

TABLE IV

Effects of oxygen at 3 atmospheres absolute on development of tubularian embryos

Before expt.		Treatment	At 48 hours						
Stage	No.		Morula	Polygon & star	Basket	Actinula		Polyp	Dead or lost
						Early	Late		
Morula	35	Control	0	0	0	1	*12*	1	21
	35	O_2–3AA	0	5	*21*	1	0	0	8
Star (2 lots)	95	Control	0	0	0	7	*26*	17	45
	95	O_2–3AA	0	9	*44*	29	0	0	13
Totals	130	Control	0	0	0	8	*39*	18	65
	130	O_2–3AA	0	5	*74*	30	0	0	21

the basket stage at 48 hours. Fifteen (62.5%) of the surviving controls were late actinulae at the time they were recorded.

Oxygen at 3 and 4 atmospheres (Tables IV and V) inhibits the later differentiation of the embryos with the majority (67.8%) arrested at the basket stage and most of the remainder at the early actinula stage. There were two late actinulae and no polyps. Indeed, it appeared that the larvae were unable to attach to the substrate because of deficiency in adhesive organ development.

TABLE V

Effects of oxygen at 4 atmospheres absolute on tubularian embryos in pasteurized seawater*

Before expt.		Treatment	At 48 hours						
						Actinula			
Stage	No.		Morula	Star	Basket	Early	Late	Polyp	Dead or lost
Morula	50	Control	0	1	14	*24*	0	0	11
	50	O_2–4AA	*21*	15	6	0	0	0	8
Star	50	Control	—	0	5	*19*	8	5	13
	50	O_2–4AA	—	10	*36*	2	0	0	2
Basket	50	Control	—	—	0	17	*20*	10	3
	50	O_2–4AA	—	—	15	*32*	0	0	3
Actinula	50	Control	—	—	—	2	6	*42*	0
	50	O_2–4AA	—	—	—	*32*	15	1	2
Totals	200	Control	0	1	19	*62*	34	*57*	27
	200	O_2–4AA	22	25	*57*	*66*	15	1	14

* 20 minutes at 70° C.

To test whether the inhibition was reversible or whether it reflected merely a moribund condition, 18 of these embryos, 6 each in early and late basket and early actinula stages, were removed from the chamber at 48 hours and were permitted to recover in the cold room. After 24 hours there were 2 late actinulae and 16 polyps. Because of the small numbers groups of 50 embryos were tested. This series (Table VI) demonstrates inhibition by 4 A.A. of oxygen similar to that shown in Table V. In addition, after 24 hours in sea water at normal pressure the oxygenated embryos were still in the early actinula stage although the controls had continued to develop. However, by 36 hours all 48 oxygenated embryos had become attached polyps. Again, the embryos tested in the basket stage showed fewer deaths than did the morulae.

In most of the experiments records were kept throughout an extended period. Table VII is a summary of one of the two typical experiments with 2½ atmospheres and illustrates the fact that there was an appreciable time lag in the inhibition produced by excess oxygen. No evidence of inhibition was seen after 24 hours of treatment. However, by 48 hours blockage was nearly 100% in the hyperbaric

TABLE VI

Reversibility of inhibition of development by removal from hyperbaric oxygen

Stage before expt.	Treatment	At 48 hours						
		Morula	Star	Basket	Actinula		Polyp	Dead or lost
					Early	Late		
Morula	Control	0	0	0	0	2	0	*48*
	O_2–4AA	*28*	10	8	1	0	0	3
Basket	Control	0	0	0	0	*31*	12	7
	O_2–4AA	0	0	0	*48*	0	0	2
		24 Hours after Removal						
Morula	Control	0	0	0	0	0	0	*50*
	Air	*15*	12	9	1	0	0	13
Basket	Control	0	0	0	0	1	*37*	12
	Air	0	0	0	*48*	0	0	2
		36 Hours after Removal						
Morula	Control	All dead						
	Air	Unchanged—beginning to disintegrate						
Basket	Control	0	0	0	0	0	*20*	30
	Air	0	0	0	0	0	*48*	2

embryos and larvae. By contrast, the controls continued development. Twelve out of fifteen surviving controls had transformed into polyps by 76 hours and all but one had transformed by 116 hours.

Embryos and larvae from both control dishes in air and experimental in hyperbaric oxygen were tested for succinic dehydrogenase activity using the modification of the method of Nachlas *et al.* (1957) mentioned in Material and Methods. Estimations of activity are summarized in Table VIII. The controls exhibited a progressive increase from polygon to late actinula in overall staining with the blue formazan, as had been observed earlier (Miller *et al.*, 1964), with localized centers of activity shown by each group of organs when they began to differentiate. The most prominent of these were used for rating the concentration of the formazan on a scale of – to ++++.

Below is a list, in order of their appearance, of the regions of increased succinic dehydrogenase activity: (1) the endoderm at the tips of the developing proximal tentacles (from the end of the morula stage through early actinula), (2) the ectoderm around the bases of the proximal tentacles (from star to late actinula), (3) the ectoderm of the perisarc-secreting zone (from late basket through polyp), (4) the ectoderm of the future gonophore region (from early actinula through

TABLE VII

Development of inhibition by hyperbaric oxygen (2½ AA)

Treatment	Time (hours)	Morula	Polygon + tail	Basket	Actinula		Polyp	Dead or lost
					Early	Late		
Control	24	7	6	7	0	0	0	0
Oxygenated		4	9	7	0	0	0	0
Control	48	2	1	0	2	*11*	0	4
Oxygenated		4	3	*10*	3	0	0	0
Control	76	0	0	0	2	1	*12*	5
Oxygenated		4	2	*10*	3	0	0	1
Control	116	0	0	0	1	0	*13*	6
Oxygenated		3	4	*8*	2	0	0	3

20 Morulae in each lot, stages recorded at 24 hour intervals.

polyp), and (5) the ectoderm of the holdfast (from early actinula through late actinula).

By contrast, the embryos which were subjected to hyperbaric oxygen showed less overall development of the blue formazan, and were particularly deficient in the

TABLE VIII

Effects of hyperbaric oxygen at 3 atmospheres absolute upon succinoxidase activity

Stages		Endoderm of tentacles	Ect. at base of tentacles	Ect. of gonophore region	Ect. of perisarc zone	Ect. of holdfast zone
Polygon	Control	++	–	–	–	–
	Oxygenated	+	–	–	–	–
Basket	Control	+	++	–	+	–
	Oxygenated	–	–	–	–	–
Early Actinula	Control	+	++	++	+++	++
	Oxygenated	–	–	–	–	–
Late Actinula	Control	–	++	+++	++++	++
	Oxygenated	–	–	–	+ ?or –	–
Attached (hydranth + stem)	Control	–	++	+++	++	–
	Oxygenated*	0	0	0	0	0

* Did not reach this stage.

TABLE IX

Effects of increased pressure (nitrogen added—oxygen unchanged)

Before expt.		Treatment	At 48 hours						
Stage	No.		Morula	Star	Basket	Actinula		Polyp	Dead or lost
						Early	Late		
		2 A.A. of 10% O_2 + 90% N_2							
Morula	75	Control	0	0	1	10	14	*36*	14
	75	2 A.A.	0	0	0	14	8	*27*	26
Basket	25	Control	0	0	0	1	0	*22*	2
	25	2 A.A.	0	0	0	1	1	*23*	0
		1 Atmosphere Air + 3 Atmospheres N_2							
Morula	50	Control	15	6	8	0	0	0	*21*
	50	4 A.A.	10	12	0	0	0	0	*28*
Basket	50	Control	0	0	0	0	8	*34*	8
	50	4 A.A.	0	0	0	0	13	*28*	9

loci of intense staining which accompanied the differentiation of intense staining which accompanied the differentiation of the controls. The few larvae that reached late actinula stage showed slight evidence of activity of succinic dehydrogenase in the perisarc-secreting zone (rated +) but in all cases this was far less than that in controls in which the staining was the most intense of any region at any stage of development (rated ++++, Table VIII).

Two experiments were performed to determine whether pressure itself influenced these results. In the first the experimental embryos were exposed to 2 atmospheres of 10% O_2 + 90% N_2 and in the second, to 1 atmosphere of air plus 3 atmospheres of nitrogen. In Table IX it is seen that with normal oxygen and pressure increased with nitrogen there was no difference between experimental and control development. Also, there was no evidence of nitrogen narcosis in this material.

DISCUSSION

These experiments indicate that Barth's highest oxygen levels were very near those which are inhibitory and that at pressures between 1 and 2 atmospheres the toxic effects of oxygen begin to overbalance the stimulatory effects.

Oxygen toxicity has been recognized ever since the classic studies of Paul Bert (1878), and the basic concepts regarding its action are little changed today from those he proposed over 90 years ago. From his extensive studies on bacteria, plants and lower animals as well as mammals, he concluded that elevated pressures of oxygen were toxic because they inhibited metabolic activities of cells. A detailed review of work in this field was published in 1968 by Haugaard.

The inactivation of succinic dehydrogenase found in our histochemical studies confirms for a hydroid the biochemical findings on mammals (Lehman, 1935; Libbrecht and Massart, 1937; and Stadie and Haugaard, 1945). Although Libbrecht and Massart (1937) found inactivation of succinic dehydrogenase, they attributed this to be secondary to the effects on cytochrome oxidase and the formation of "oxygen actif" which has not been confirmed by later studies. Stadie and Haugaard (1945), who made a thorough study of the situation, found that the mechanism of inactivation is that of oxidation of the active sulfhydryl groups to the inactive thiol form. They reported that the inactivation was reversible and that reactivation of the enzyme could be achieved by incubation with cysteine or glutathione. In Table VI is documented that fact that although the blockage of development of *Tubularia* by 4 atmospheres of pressure was unchanged at 24 hours after removal from the chamber the embryos had recovered when observed at 36 hours. It will be of interest to determine if the inhibition of development and the inactivation of succinic dehydrogenase in tubularian embryos and larvae can be prevented by appropriate administration of the reduced SH compounds glutathione and cysteine.

The lag period of 24 hours which was found before the appearance of symptoms is a characteristic of oxygen toxicity as studied in mammals. This together with its reversibility upon return to atmospheric pressures, even at an advanced stage, is consistent with the hypothesis that the toxicity of hyperbaric oxygen is caused by the oxidation of certain essential enzymes or cofactors. It is assumed that under normal conditions these are present in excess and that they can be reactivated or resynthesized rapidly as soon as oxygen tensions fall to normal levels (Haugaard, 1965). It is possible that in these very small embryos the reactivation may be inadequate in some cases for development to continue.

The protection against death which was noted in these experiments requires comment. Hyperbaric oxygen has been shown to inhibit the growth of bacteria and of protozoa (Caldwell, 1965; Elliott, Travis and Bak, 1962; Towers and Hopkinson, 1965). Since a wide variety of these organisms inhabits *Tubularia* colonies and some are known to inhibit regeneration or to be parasitic, it is possible that inhibition of proliferation of these would result in the survival of greater numbers of embryos in the hyperbaric chamber.

On the other hand, the egg of *Tubularia* does not contain a large amount of stored energy in the form of yolk. As a result the possibility also exists that the inhibition of enzyme activity, as indicated by the succinic dehydrogenase estimations, prevented the early exhaustion of energy stores and thereby prevented death from inanition.

Finally, the fact should be emphasized that hyperbaric oxygen, which so completely (but reversibly) blocks differentiation, has no demonstrable effect upon cytochrome oxidase, carbonic anhydrase and many other enzymes (Haugaard, 1965). On the other hand this treatment has been shown to inactivate the free sulfhydryl groups of the SH-enzymes including succinic dehydrogenase.

It was concluded from the present study that hyperbaric oxygen blocks succinic dehydrogenase activity in developing *Tubularia,* and that since the parallelism between inhibition of differentiation and the reduction in succinic dehydrogenase activity is so precise, that there is a high degree of probability of a cause-effect relationship between the two groups of data.

These results have significance in the analysis of development of this species since they implicate certain groups of enzymatic processes as having key roles in certain aspects of differentiation. By combining biochemical inhibition or stimulation with blockade of enzymatic processes by hyperbaric oxygen a clearer understanding of embryonic differentiation may be anticipated.

SUMMARY

1. Hyperbaric oxygen reduced deaths of tubularian embryos and larvae in standing seawater.

2. Hyperbaric oxygen blocked differentiation at pressures of 2, $2\frac{1}{2}$, 3 and 4 atmospheres absolute. At $1\frac{1}{2}$ atmospheres it had little or no effect.

3. The blockade occurred between 24 and 48 hours after early actinula or late basket stages were introduced into the chamber. The blocked embryos were unable to secrete holdfast material or produce perisarc.

4. After 48 hours at 4 atmospheres the block was usually completely reversible during 24 to 36 hours after removal. Even after 72 hours a few blocked larvae attached and transformed into polyps when removed from the hyperbaric chamber.

5. Histochemical visualization showed an overall reduction of succinic dehydrogenase activity in all embryos subjected to hyperbaric oxygen. Localized zones of high activity, which in controls were associated with the differentiation of tentacles, gonophore buds, perisarc-secreting region and adhesive organ, failed to develop.

6. Since hyperbaric oxygen reversibly blocks certain enzyme systems but has no effect upon others, this method can prove useful in the biochemical analysis of developmental processes.

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ASSIMILATION OF PHOTOSYNTHETIC PRODUCTS OF ZOOXANTHELLAE BY A REEF CORAL[1]

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All hermatypic, or reef building, corals possess autotrophic endosymbiotic algae (zooxanthellae) within their gastrodermal cells. There has been much interest in and controversy about the nature and extent of the contribution of the algae to organic productivity of the coral animal and the reef community (see reviews of Yonge, 1963, and McLaughlin and Zahl, 1966). Ecological studies on some Pacific coral reef communities infer that symbiotic algae in corals are important producers on the reef (Sargent and Austin, 1949, 1954; Odum and Odum, 1955; Kohn and Helfrich, 1957). Measurements of the ratio of photosynthesis to respiration in several coral species from the Pacific and the Caribbean imply that the production of reduced organic carbon by zooxanthellae is sufficient to more than offest losses through respiration (Kanwisher and Wainwright, 1967; Roffman, 1968). This raises the question as to how organic material produced by zooxanthellae might be acquired by the host coral? The most likely alternatives appear to be that algae are digested by the çoral; or that algae are released by the coral and returned to it via the predator food chain; and/or that soluble photosynthate may be translocated *in situ* from the algae to the host. We have investigated the latter alternative.

Selective release and translocation of soluble photosynthate by symbiotic algae has been demonstrated experimentally in other coelenterates such as green hydra (Muscatine and Lenhoff, 1963), sea anemones (Muscatine and Hand, 1958; Trench, 1968) and zoanthids (von Holt and von Holt, 1968), in associations of algae and molluscs (Goreau, Goreau, and Yonge, 1965) and in lichens (Smith and Drew, 1965; Drew and Smith, 1967). Goreau and Goreau (1960) attempted to detect translocation of photosynthate from zooxanthellae to host corals by autoradiography. Their conclusion, that little translocation occurred after 50 hours incubation with $^{14}CO_2$, was based on observation of relatively few exposed grains in autoradiographs of sections of coral tissue (minus skeleton). It is is now recognized that the preparation of thin sections removes most of the soluble material from the tissues and the extent of translocation may easily be misinterpreted from autoradiographs, especially if much of the material translocated remains in the host in a soluble form. Von Holt and von Holt (1968) have presented evidence which they interpret as demonstrating translocation of photosynthate in the hermatypic coral *Scolymia lacera*. After incubation with $^{14}CO_2$ for three hours in the light, the pattern of incorporation of ^{14}C among various tissue fractions was noted.

The present paper describes experimental studies on the metabolism of $^{14}CO_2$, the *in situ* translocation of ^{14}C, and its assimilation by the Hawaiian reef coral

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Pocillopora damicornis. Included are data on specific labeled substrates in the algae and animal tissues, the magnitude of heterotropic fixation, the nature of the translocated material, and the acquisition of ^{14}C by the skeleton of *Pocillopora.* This genus of hermatypic coral is common among Indo-Pacific reefs. Intact corals were exposed to $^{14}CO_2$ in the light and dark for varying lengths of time up to 24 hours, and then the amount and nature of the fixed ^{14}C within the coral was ascertained. Of the total ^{14}C fixed photosynthetically by the zooxanthellae, about 35–50% is released and incorporated into host coral constituents.

METHODS

Incubation with $Na_2{}^{14}CO_3$ (Step A)

Letters in parentheses refer to steps in the experimental protocol outlined in Figure 1.

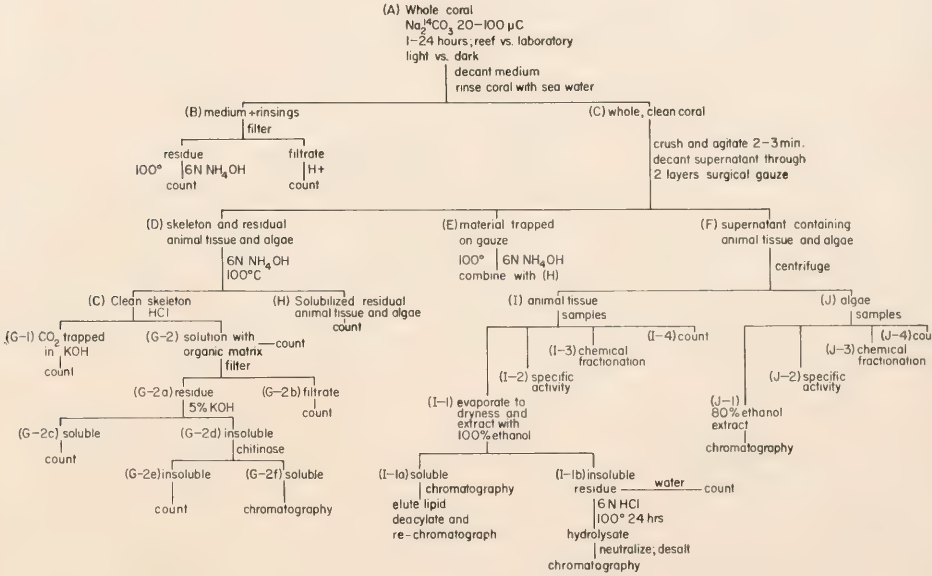
(A) Whole coral
Na$_2^{14}$CO$_3$ 20–100 μC
1–24 hours; reef vs. laboratory
light vs. dark
decant medium
rinse coral with sea water
(B) medium + rinsings
filter
residue
100°
6N NH$_4$OH
count
filtrate
H+
count
(C) whole, clean coral
crush and agitate 2–3 min.
decant supernatant through
2 layers surgical gauze
(D) skeleton and residual animal tissue and algae
6N NH$_4$OH
100°C
(E) material trapped on gauze
100°
6N NH$_4$OH
combine with (H)
(F) supernatant containing animal tissue and algae
centrifuge
(C) Clean skeleton
HCl
(H) Solubilized residual animal tissue and algae
count
(G-1) CO$_2$ trapped in KOH
count
(G-2) solution with organic matrix
count
filter
(G-2a) residue
5% KOH
(G-2b) filtrate
count
(G-2c) soluble
count
(G-2d) insoluble
chitinase
(G-2e) insoluble
count
(G-2f) soluble
chromatography
(I) animal tissue
samples
(I-1) evaporate to dryness and extract with 100% ethanol
(I-2) specific activity
(I-3) chemical fractionation
(I-4) count
(I-1a) soluble
chromatography
elute lipid
deacylate and
re-chromatograph
(I-1b) insoluble residue
water
count
6 N HCl
100° 24 hrs
hydrolysate
neutralize; desalt
chromatography
(J) algae
samples
(J-1) 80% ethanol extract
chromatography
(J-2) specific activity
(J-3) chemical fractionation
(J-4) count

FIGURE 1. Summary of procedures used to fractionate whole labeled corals.

(*a*) *Laboratory experiments. Pocillopora damicornis* was collected from the vicinity of Checker Reef in Kaneohe Bay, Oahu, Hawaii at depths ranging from 1–5 meters. Small heads (30–40 g fresh wt) were dislodged from the substrate by gentle tapping at the base with a geological hammer. Epifaunal organisms were removed and the corals were transported to the laboratory in buckets of sea water. Some corals were maintained at the laboratory submerged in shallow tanks of running sea water pumped from the Bay, and used within a day after collection. Others were immediately placed in a beaker with 200 ml Millipore-filtered sea water to which 25–100 μc $Na_2{}^{14}CO_3$ (1 μc/μl) were added. Beakers were covered with parafilm, placed in a water bath controlled at 25 ± 1.0° C and incubated for 24 hours. The water bath was constructed of ¼ inch plexiglass and placed above a bank of fluorescent lights delivering 2000 ft-c to the bottom surface of the beaker. Experiments of 1–6 hours were performed only under constant illumination while 24-hour incubations were performed either under constant light or constant darkness. In dark control experiments the isotope was added in a dark room, the beakers were wrapped in aluminum foil and then placed in the water bath.

(*b*) *Reef experiments.* Incubations were carried out *in situ* on the reef for 24 hour periods, starting at 10:00 AM. In initial trials small heads attached to the substrate were enclosed with transparent plastic bags and observed at intervals over 48 hours. Normal underwater surge caused the bags to sway and to chafe the tips of the stationary corals, and bare skeleton was exposed after 24 hours. This method was abandoned in favor of an alternative. Small heads were gently dislodged from the substrate and placed in a plastic bag. The bag was squeezed free of all but about 200 ml sea water and a sealed 10 ml screw cap vial containing 100 μc isotope placed inside the bag. The bag was then tied shut and suspended at the site from which the coral was originally taken, usually at a depth of 3–4 meters. To release the isotope we manipulated the bag so that the vial inside could be grasped and the cap unscrewed, allowing the isotope to mix with the incubating sea water. This incubation method avoided chafing since the bag and coral moved together. The coral, however, was still at its preferred depth and location and at ambient temperature (*ca.* 26° C). The operations were done using SCUBA. After incubation the bag was wrapped in aluminum foil, brought to the surface, and deposited in the laboratory within 20 minutes. After all incubations, either in the laboratory or on the reef, the incubation medium was decanted and the corals rinsed several times with clean sea water. Medium and rinsings (B) were passed through a Whatman No. 1 paper filter. The filtered residue was dissolved in hot 6N ammonium hydroxide, the filtrate was acidified to remove unused carbonate-^{14}C and both fractions were assayed for radioactivity.

Experimental procedures: Fractionation of ^{14}C-labeled corals (*Step C* et seq.)

After incubation, the rinsed, ^{14}C-labeled coral heads were broken into small pieces and placed in an aluminum foil envelope. The broken base of the thick, central branch of each coral head, exposed to the sea water during the incubation, was discarded to avoid error due to labeling by exchange. The remaining coral was crushed with a hammer, quantitatively transferred to a 250 ml flask, and the coarsely fragmented slurry shaken vigorously by hand for 2 minutes. The agitation and abrasive action of the coral chips yielded a brownish supernatant containing algae and animal tissue which was then decanted through several layers of surgical gauze into 12 ml graduated centrifuge tubes. The gauze filter and fragments remaining in the flask were rinsed briefly with clean sea water and the rinsings combined with the supernatant. This treatment yielded two major fractions: a supernatant fraction (F) containing the algae and animal tissue; and a residual fraction (D), left behind in the flask, containing the skeleton and a portion of the algae and animal tissue which could not be freed from the skeleton by the mechanical abrasions. Combined with fraction (D) was the material trapped on the filter (E).

(*a*) *Separation of algae and animal tissue* (*Step F*) Immediately upon obtaining the supernatant mixture of algae and animal tissue, it was centrifuged at 3000 rev/min (International Clinical Centrifuge Model CL). The supernatant suspension of animal tissue was drawn off and saved, and the algal pellet was resuspended in clean sea water and washed twice by centrifugation. The process from abrasion of whole coral to separated algal and animal components took about 5 minutes. Rapid separation is essential since the algae can be expected to release organic material when suspended in the homogenized animal tissue (Muscatine, 1967) (See Discussion). The washings were combined with the animal tissue fraction (I) which was adjusted to known volume and counted (I-4). The algae (J) were suspended in hot 80% alcohol, adjusted to known volume and assayed for radioactivity (J-4). The total activity of the separated algal and animal fractions from each experiment was used in the calculation of per cent translocation in that experiment.

To aid in the estimation of efficiency of separation of algae and animal tissue, mouse liver protein, labeled with ^{35}S as described by Lenhoff (1961), was employed as a tracer (See Results).

(*b*) *Chemical fractionation of tissue* (*Steps J-3, I-3*) Samples of coral tissue and algae were each fractionated by differential solubilities using the method of Roberts, Abelson, Cowie, Bolton and Britten (1955) as modified by Lenhoff (1961). Four major fractions were obtained by sequential treatment with cold 5% trichloroacetic acid (Cold-TCA); acidified 80% ethanol (Alc-sol); and hot 5% TCA. The final fraction was the material insoluble in TCA and alcohol (Alc-TCA-Insol.). This fractionation procedure was used only for quantitative estimation of the fate of labeled carbon in the algae and animal tissues. For qualitative analysis, animal tissue, freshly separated from the algae, was evaporated to dryness and extracted twice with 10 ml hot dry absolute ethanol (I-1). The virtually salt-free ethanol-soluble extract was processed for radiochromatographic analysis (I-1a). The alcohol-insoluble residue (I-1b) was resuspended in water to dissolve salt, was centrifuged and the aqueous supernatant assayed for a radioactivity and then discarded. The remaining salt-free alcohol-water insoluble residue was hydrolyzed with 6 N HCl at 106° C for 24 hours in a sealed tube. The resulting hydrolysate was diluted with several volumes of distilled water, evaporated to dryness, taken up in absolute ethanol and saved for radiochromatographic analysis. The algae were extracted (J-1) and analyzed in an identical fashion except that insoluble material was not hydrolyzed.

Experimental procedures: Assay of skeletal $^{14}CO_3^=$ *and organic matrix*-^{14}C (*Step D* et. seq.)

(*a*) *Assay of residual animal tissue and algae* Skeletal fragments comprising the residual fraction (C) were boiled in 6 N NH_4OH to dissolve and remove residual animal tissue and algae. The tissue-free skeleton (G) was washed in distilled water. The washings were combined with the dissolved organic material (H), and an aliquot of this material was counted. From the radioactivity in fractions (H), (E), (J-4), and (I-4) the total radioactivity in the coral tissues (residual tissues plus separated animal tissue and algae) in each experiment was ascertained.

(*b*) *Assay of skeletal* $^{14}CO_3^=$ Clean skeleton (G) was dissolved in cold 6 N HCl in an evacuated flask (see Doty and Oguri, 1959) and the carbon dioxide collected in 8 N KOH. The KOH fraction containing the $^{14}CO_2$ (G-1) was counted.

(*c*) *Analysis of the skeletal organic fraction* The HCl fraction containing the skeletal organic material (G-2) was counted. The insoluble portion was collected by filtration and washed several times with distilled water. Filtrate and washings were assayed for radioactivity (G-2b).

The washed insoluble material (G-2a) was assumed to be chitin, the chief constituent of the matrix in *P. damicornis* (Wainwright, 1963). This material was suspended in 4 volumes of 5% (w/v) KOH and boiled to dissolve nonchitinous organic constituents (Reynolds, 1954). Soluble material (G-2c) was counted. The insoluble chitin (G-2d) was washed until the effluent was neutral, solubilized and re-precipitated, and incubated with chitinase (Calbiochem #220471) according to the method of Skujins, Potgeiter and Alexander (1965). The reaction was stopped by boiling. After centrifugation, the supernatant (G-2f) was taken to dryness under reduced pressure, then taken up in hot 80% ethanol and co-chromatographed with authentic glucosamine and N-acetyl glucosamine. The insoluble material remaining after chitinase hydrolysis (G-2e) was counted.

Analytical procedures: Total protein

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin standards (Armour and Co.).

Analytical procedures: Radiochromatography and assay of radioactivity

All tissue fractions which were assayed for radioactivity are indicated in Figure 1. Aliquots of known volumes of material were assayed by conventional planchet and liquid scintillation techniques with corrections for background. Two-dimensional chromatography was carried out on soluble samples using Whatman no. 4 paper and phenol : water (100 : 39, w/v) in the first dimension and butanol : propionic acid : water (1246 : 620 : 884 v/v/v) in the second dimension. Radioactive compounds were located on chromatograms with Kodak Blue-sensitive medical x-ray film as described by Bassham and Calvin (1962).

Analytic procedures: Lipid deacylation (I-la)

Ethanol-soluble lipids from animal tissues were eluted from paper chromatograms with a mixture of absolute ethanol and 10% (v/v) toluene. An aliquot was counted and the remainder was dried under nitrogen. To the residue, 100 μl 0.2 *M* KOH in dry methanol (equal volumes) was added. The suspension was incubated at 35° C for 30 minutes and then water added until a white precipitate appeared. Nine mg Dowex 50 H^+ (200–400 mesh) was added with shaking followed by 1 ml diethyl-ether. Centrifugation yielded a biphasic suspension. The upper layer containing the nondeacylated material was counted and discarded. The bottom layer was taken up in 0.5 ml of the phenol : water solvent and chromatographed.

Analytical procedures: Identification of unknowns

Unknown radioactive compounds were provisionally identified by cochromatography as described previously (Muscatine, 1965, 1967).

RESULTS

^{14}C fixation by intact corals in light and dark

Table I shows that intact corals fixed $^{14}CO_2$ in the light and in the dark. Over a 24 hour period the amount of fixation by corals on the reef was significantly higher than in the dark controls. Fixation in the dark was about 10–12% of that in the light. Evidence given below and in Table I shows most of the ^{14}C fixed in the light over a 24 hour period accumulated in the coral tissues and only 9–12% in the skeleton. It is concluded that in the light, the bulk of the ^{14}C fixed in the living tissues was the result of photosynthesis by the zooxanthellae, and that a smaller proportion was fixed heterotrophically.

TABLE I

Distribution of total ^{14}C in major constituents of P. damicornis *in three 24-hour incubations*

	Reef-24 hours				Dark-24 hours	
	cpm	%	cpm	%	cpm	%
Medium	92,000	0.9	58,000	0.7	4,500	0.6
Animal tissue*	4,349,000	45.0	2,463,270	32.0	200,889	25.7
Algae*	4,268,000	44.2	4,281,770	55.5	203,186	26.1
Skeleton**	(28.2g)		(26.9g)		(29.1g)	
As carbonate†	927,000	9.6	884,930	11.5	336,150	47.0
As matrix†	17,065	0.2	16,220	0.2	4,147	0.5

Corals of approximately the same dry skeletal weight were incubated in equal volumes of sea water containing $100 \mu C$ $Na_2{}^{14}CO_3$.

* These totals include radioactivity in the residual tissue which was assayed and added to the already separated animal tissue and algae in their respective proportions. Proportion of radioactivity in animal tissue given in Table IV, data for 24 hours.

** Total skeletal ^{14}C was determined from the product of skeletal weight (in parentheses) and the average specific activity (cpm/g skeleton). The average specific activity was calculated from data for 24-hour incubations in Table VI.

† The proportion of ^{14}C as carbonate is the difference between the total skeletal ^{14}C and the matrix ^{14}C. Matrix ^{14}C was calculated from the data for average percent of ^{14}C in the matrix in 24-hour incubations in Table VI.

In all experiments with intact corals less than 1.0% of the total fixed ^{14}C was detected in the incubation medium. This exogenous organic radiocarbon may have originated either from labeled coral mucus, from bacteria residing on the surface of the coral, from excreted soluble metabolites, or from discharged zooxanthellae.

Removal of coral tissue from skeleton

To separate the algae and animal tissue, the tissues first had to be removed from the skeleton. Mechanical abrasion as described under Methods removed most of the living tissues but some tissue was invariably retained in the residual fraction (D). Table II shows that of the total labeled coral tissue in each of 18 experiments a mean value of 52 ± 11% was obtained for analysis. There are no

Table II

Recovery of ^{14}C-labeled tissue (animal plus algae) from P. damicornis *homogenates*

Incubation time (hours)	Recovery (%)
Laboratory (light)	
1	30,47
2	60,46
3	47,39
4	53,53
5	50,55
6	43,48
24	63,70
Reef (ambient light)	
24	81,37
Laboratory (dark)	
24	54
	$\bar{X} \pm$ S.D. = 52 ± 11

The total labeled tissue was the sum of the recovered tissue (F) plus the residual tissue (D plus E). Per cent recovery was then calculated as (recovered/recovered + residual) × 100.

obvious or significant correlations between amount of tissue recovered and incubation conditions.

Efficiency of separation of algae and animal tissue

Examination of suspensions of algae separated from animal tissues by centrifugation revealed traces of non-algal debris such as nematocycts, *etc.* Occasionally, algal cells could be found in the animal tissue fraction. To estimate accurately translocation of ^{14}C from algae to animal it was essential first to measure the extent of mutual contamination of each fraction.

To determine the extent to which the animal fraction contaminated the algae, finely homogenized ^{35}S-labeled mouse liver was added to an unlabeled coral homogenate to serve as a tracer for "animal tissue." After centrifugation, in three trials, 6.1, 6.8 and 5.0 per cent of the labeled liver added was recovered in the algal pellet. To determine the extent to which the algae contaminated the animal fraction we had planned to add a suspension of labeled zooxanthellae to a "cold" homogenate, centrifuge to pellet the algae and then assay the supernatant for radioactivity. But since coral homogenate stimulates excretion by the algae (Muscatine, 1967), this approach had to be modified by adding the labeled algae to boiled homogenate, in which the stimulatory activity is destroyed. In two trials, 0.5 and 2.0 per cent of the total activity added was recovered in the supernatant. From these data we conclude that the separation of algae and animal tissue from *P. damicornis*, as described here, is about 95% efficient and mutual contamination of fractions does not constitute significant error, either in the estimation of translocation or in the qualitative analysis of the fractions.

Proportion of animal to algal tissue

To estimate accurately the magnitude of translocation it was important to determine the variation in the relative amounts of algae and animal tissue in representative samples. Table III shows that ratios of animal to algae protein ranged from 0.61 to 1.59. This wide range was not entirely unexpected since removal of tissue from the skeleton was incomplete, and numbers of algae per unit coral tissue vary among colonies of *P. damicornis* and other coral species (our unpublished observation; Goreau, 1963). The interpretation of this variation and its treatment in estimating translocation is given below.

TABLE III

Relative amounts of animal and algae protein in samples recovered from whole coral

Coral sample wet weight (grams)	Animal fraction mg protein/sample	Algal fraction mg protein/sample	Animal protein/ Algal protein
31.5	3.16	2.68	1.18
27.0	3.26	2.81	1.16
28.5	2.11	2.11	1.00
27.0	3.26	2.05	1.59
31.0	2.11	3.07	0.69
25.0	2.11	3.07	0.69

Photosynthesis and release of photosynthate by algae in vitro

It has already been shown that zooxanthellae isolated from *P. damicornis* can release up to 40% of their photosynthate when in the presence of homogenate of their host tissues (Muscatine, 1967). These preliminary studies were confirmed here. Figure 2 and 3 are, respectively, autoradiographs of chromatograms of the medium and of an 80% ethanol-soluble cell extract of zooxanthellae incubated in the light for 2 hours with host homogenate and $Na_2{}^{14}CO_3$. Figure 2 shows the major products released by the cells. The relative proportions are glycerol, 82%; glycolic acid, 6.4%; alanine, 1.7%; glucose, 0.7%; unknown, 8.9%. The very striking difference in chromatographic pattern of ^{14}C in the medium and in the cell extract (Figure 3) illustrates that glycerol is released by a selective process and not by cell lysis. If the cells had lysed, a wide range of water soluble intracellular compounds would appear in the medium. As noted in the preliminary study, the relative proportions of material released to the medium may vary, especially in incubations of more than 2 hours. In such incubations glycolic acid and other organic acids are detected in greater abundance, and in the presence of host homogenate, the excreted glycerol may be incorporated into lipid *in vitro*. The host homogenate alone neither manufactures ^{14}C-glycerol nor fixes appreciable amounts of $^{14}CO_2$. Finally, the pattern of photosynthetic products of the algae *immediately after isolation* as shown in Figure 3 is virtually identical with those of algae labeled *in vitro* (Fig. 1, fraction J-1). Identification of the labeled intracellular constituents was limited to glucose, the major carbohydrate, alanine and glycerol. Intracellular ^{14}C-glycerol in Figure 3 is 6.6% of the total soluble ^{14}C detected within the cells.

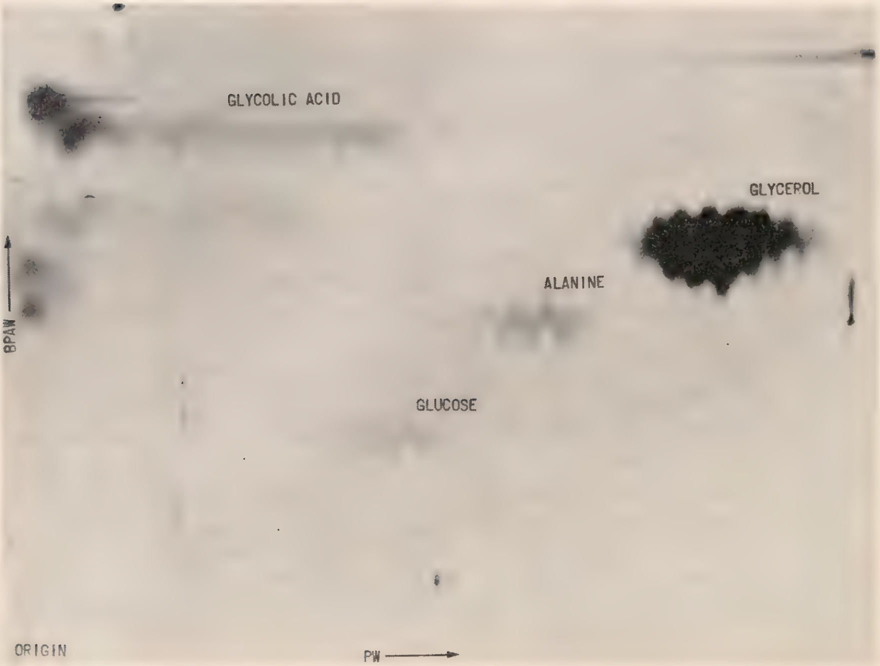

FIGURE 2. Radioautograph of chromatogram of medium from zooxanthellae incubated in light with $Na_2{}^{14}CO_3$, for 2 hours, showing labeled soluble extracellular products.

Translocation of fixed ^{14}C from algae to animal tissue in vitro

Translocation has been measured in other associations as that per cent of the total ^{14}C fixed which is detected in the heterotroph. Measurement of translocation in reef corals is not as straightforward since the skeleton also acquires ^{14}C in the carbonate and in the organic matrix. Data from light and dark experiments in Table IV suggest that some of the activity in the skeletal components could be acquired through fixation by the algae and translocation, and some by direct assimilation of $^{14}CO_2$ from the environment. To simplify our interpretation, and until the source of ^{14}C in the skeleton can be defined critically, we express translocation as that fraction of the total ^{14}C in the separated tissues which is detected in the animal tissue only, omitting from consideration the skeletal ^{14}C. This parameter is distinct from per cent distribution of ^{14}C as expressed in Table I. The amount of ^{14}C in animal tissue as a function of incubation time is shown in Table IV. Even after one hour incubation appreciable ^{14}C is detected in the animal tissue. The precentage rises to a maximum of almost 42% after 24 hours in laboratory incubations (constant illumination) and 36–50% in the animal tissues of corals incubated for 24 hours on the reef.

The appearance of such relatively large amounts of ^{14}C in the animal tissue is taken as evidence for translocation of ^{14}C from algae to animal *in vivo*. Comparison of relative tissue specific activities (cpm/μg protein) in Table IV gives an alternative estimate of translocation, and one which is independent of variation in

TABLE IV

Estimates of in vivo *translocation of* ^{14}C

Incubation time (hours)	Radioactivity in animal tissue (%)	Specific activity (cpm/μg protein)	
		Animal	Algae
Laboratory (light)			
1	14.6	86	361
2	19.5	296	681
3	23.2	541	1180
4	26.9	533	1451
5	34.5	1573	1624
6	34.7	1218	939
24	41.8	*	*
Reef (ambient light)			
24	50.3, 36.5	*	*
Laboratory (dark)			
24	49.7	27	116

Radioactivity in animal tissue was estimated as a percentage by (cpm animal/cpm animal + algae) × 100. Asterisk denotes data not obtained.

sample mass observed in Table III. However, the specific activity determinations assume that algae and animal tissues each have a relatively constant protein content per unit volume. We have not verified this experimentally for *Pocillopora.* The specific activity data indicate that the amount of ^{14}C increases steadily in both tissues through five hours of incubation. A slight decrease in activity was observed in the 6-hour samples. About 50–55% of the fixed $^{14}C/\mu g$ protein was detected in the animal tissue after this time. This value compares favorably with the 50% level of excretion exhibited by freshly isolated zooxanthellae (Muscatine, 1967).

Compared to corals incubated in light, the dark controls in Table IV exhibit relatively low specific activity after 24 hours, confirming that only a small fraction of the ^{14}C in the animal tissue of illuminated corals is due to heterotrophic fixation, and implying that in the light most of the fixed ^{14}C in the tissues is the result of algal photosynthesis and translocation. This interpretation also assumes that in the light the algae do not stimulate heterotrophic fixation by the animal tissue.

Distribution of ^{14}C in coral tissues in light experiments

(*a*) *Qualitative analysis* (*J-1, I-1*) From 27 to 43% of the total animal tissue-^{14}C from 24-hour incubation was ethanol-soluble (I-1a). Analysis by paper chromatography revealed a large labeled lipid component and a variety of other labeled compounds, probably low molecular weight substances referable to the TCA-soluble pool (see below). In view of the role of glycerol in lipid synthesis and its involvement in translocation, it was important to ascertain which component of the animal tissue lipids were ^{14}C-labeled. After the lipid was eluted, deacylated and rechromatographed, two radioactive spots were invariably detected. One was judged to be a portion of the original lipid which was not deacylated; the

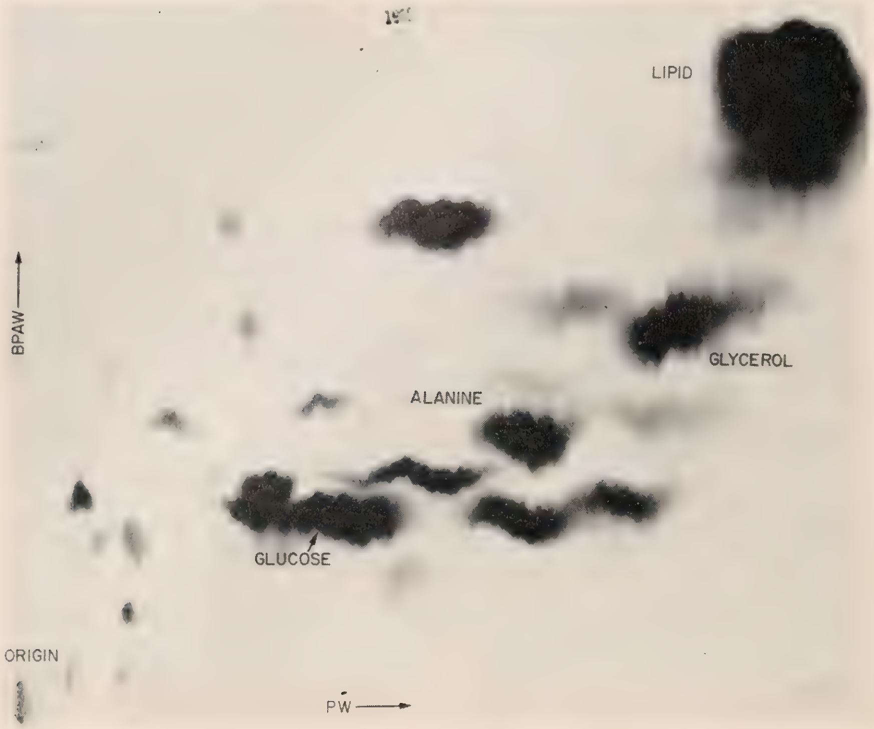

FIGURE 3. Radioautograph of chromatogram of 80% ethanol soluble extract of zooxanthellae treated as in Figure 2, showing labeled intracellular products.

other was identified as glycerol-^{14}C. No other labeled components were detected. Since most of the ^{14}C released by freshly isolated zooxanthellae is glycerol, we conclude that some glycerol-^{14}C is released *in vivo* by the zooxanthellae and then esterified with unlabeled animal compounds to form lipid.

From 56 to 73% of the animal tissue-^{14}C was ethanol insoluble (I-1b). Radiochromatographic fingerprinting of an hydrolysate of this material revealed that nearly all of the ^{14}C was ninhydrin-positive and referable to amino acids. Two compounds did not react with ninhydrin and behaved chromatographically like native and oxidized lipid, despite treatment of the insoluble fraction with hot alcohol. We conclude that the labelled ethanol-insoluble fraction of animal tissue is largely protein.

(*a*) *Quantitative analysis* (*I-3, J-3*) Table V shows the percentage distribution of ^{14}C in differentially soluble fractions of animal tissue and algae from three 24-hour reef incubations. In both tissues, the relative proportions of labeled cold TCA-soluble (amino acids, sugars, and other low molecular weight substances released by osmotic shock) and hot TCA-soluble (nucleic acids) fractions are roughly the same. It is the alcohol-soluble and alcohol-TCA-insoluble fractions which differ. In the animal tissue more than half of the activity is insoluble protein. About 20% is alcohol soluble. The reverse seems to hold for the algae,

TABLE V

Per cent distribution of radioactivity in differentially soluble fractions of animal tissue and algae after incubation for 24 hours on the reef; data from three different experiments

Fractions	Animal tissue			Algae		
	a	b	c	a	b	c
Cold TCA-soluble	19.3	19.0	18.2	16.3	14.6	18.4
Alcohol soluble	19.3	19.3	22.6	42.7	48.1	42.3
Hot TCA-soluble	4.0	3.0	3.1	6.4	12.4	15.3
Alcohol-TCA-insoluble	57.3	58.5	56.0	34.4	24.8	23.9
% recovery	89.3	91.2	94.1	76.7	86.7	98.7

with the alcohol-soluble fraction comprising the largest proportion of activity and the alcohol-TCA-insoluble fraction ranging from 25–35%. There is thus an apparent reciprocal relationship between the proportion of soluble and insoluble labeled fractions in the algae and animal tissue after 24 hours.

Distribution of ^{14}C in coral tissue from dark incubations

Table I shows the extent of ^{14}C fixation after 24 hours in the dark. Data for dark incubations in Table IV implies that both the animal tissue and the algae can acquire ^{14}C by heterotrophic fixation, or that there is some exchange of ^{14}C-labeled products of dark fixation between animal tissue and algae. The relative extent of each of these processes is not yet known. Only the animal tissue fraction was analyzed qualitatively. Labeled carbon was detected predominantly in the 100% ethanol-insoluble fraction. The radiochromatographic pattern of distribution of ^{14}C in this fraction (I-1b) after hydrolysis was qualitatively similar to the analogous animal tissue fraction in light incubations. The same ninhydrin-positive compounds referable to amino acids were detected. From this we conclude that heterotrophic fixation by *Pocillopora* contributes ^{14}C to proteins, and that this contribution is significantly augmented in the light by photosynthesis by the zooxanthellae. Due to the low activity of the soluble fraction of the animal tissue incubated in darkness, it was not analyzed. It would be interesting to know the extent to which the lipid in this fraction is ^{14}C-labeled.

^{14}C in the skeleton

Table I shows that on a percent basis, the skeleton of corals incubated on the reef for 24 hours contained about 10–12% of the total ^{14}C in the coral, while skeleton from 24-hour dark incubations accumulated 47% of the total ^{14}C. However, illuminated corals acquired 2–3 times as much ^{14}C in both the skeletal carbonate and matrix fractions as coral incubated in darkness. Table VI shows the results of analyses of small samples of cleaned skeleton after various incubation conditions. These data are based on total ^{14}C in skeleton and matrix only.

TABLE VI

Distribution of ^{14}C in skeletal carbonate and skeletal organic matrix in samples of cleaned skeleton

Incubation time (hrs)	Weight of skeleton (mg)	Total ^{14}C (cpm)	Per cent ^{14}C as $^{14}CO_3^-$	Per cent ^{14}C as organic material*
Laboratory (light)				
1	826	12,149	94.3	5.7
2	806	18,550	93.1	6.9
3	809	13,045	95.4	6.6
4	1002	33,141	95.9	6.1
24	811	23,270	96.2	7.8
Reef (ambient light)				
24	809	27,604	98.2	1.8
	806	35,444	98.8	1.2
	805	18,240	97.9	2.1
Laboratory (dark)				
24	807	7,917	99.2	0.8
	810	14,702	99.0	1.0
	803	6,495	98.6	1.4
	828	8,772	98.7	1.3

* Denotes total labeled organic material of which less than 50% may be chitin. See text for complete explanation.

Coral skeleton exhibited appreciable radioactivity after all of the tissue had been removed with alkali. Skeletons of those corals incubated in light in the laboratory for 1–4 hours or for 24 hours contained 93–96% of their ^{14}C as carbonate with specific activities between 14 and 33 cpm/mg skeleton. About 6% of the ^{14}C was acid stable and assumed to represent constituents of the organic matrix. In 24-hour reef experiments, about 98% of the activity in the skeleton was present as carbonate-^{14}C (sp. act. 22–44 cpm/mg) and only 1.2–2.1% was acid stable skeletal organic material. This slightly lower percentage of ^{14}C in the matrix in 24-hour reef experiments may result from the reef incubation which included a dark period, in contrast to the constant illumination in laboratory experiments. Even so, the absolute amount of ^{14}C in the matrix from reef incubations was still higher than that in the dark. The lowest level of incorporation of ^{14}C into skeletal carbonate (sp. act. 9–18 cpm/mg) and into organic material (1.1%) was observed in 24-hour dark incubations in the laboratory. In the dark, it is likely that ^{14}C in the organic matrix originates from heterotrophic fixation and incorporation into newly synthesized matrix. If so, then subtraction of the dark values from the light values may give a valid estimate of matrix labeling as a result of translocation of ^{14}C from the algae.

To determine, in a preliminary way, which matrix constituents were labeled, samples of skeleton from incubations in light were dissolved in cold 6 N HCl and the insoluble material remaining was collected and washed in distilled water; the washings contained negligible radioactivity. This insoluble skeletal organic material was treated and assayed as given in Figure 4. Boiling in hot KOH removed non-chitinous constitutents but the alkali treatment also dissolved about 60% of the radioactivity in the insoluble starting material. Although this alkali-soluble

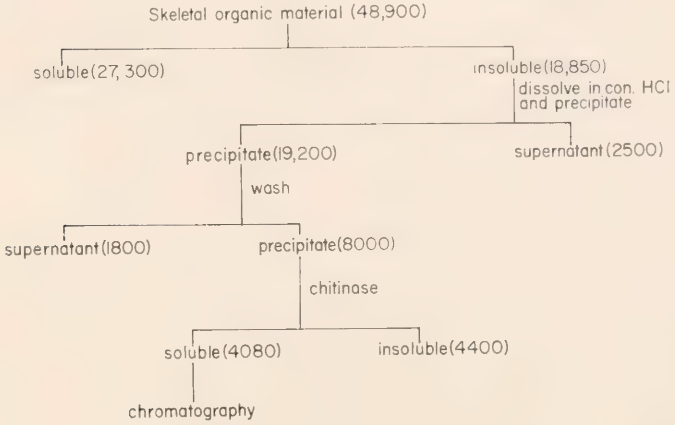

FIGURE 4. A representative flow diagram with quantitative data on analysis of matrix-^{14}C from *Pocillopora.* Numbers in parenthesis are total counts min^{-1} in the sample.

fraction was not analyzed its high activity suggests that either the matrix was contaminated with labeled coral tissue or that other constituents of the native matrix (see Discussion) were labeled and possessed high specific activity. The alkali-insoluble material, presumed to be chitin (or possibly chitosan), was hydrolyzed in a range of HCl concentrations at 100° C for times varying from 1–24 hours in a sealed tube. Under these conditions, nearly all of the activity was invariably lost from the hydrolysate. To explain this we reasoned that much of the activity in the matrix may have been associated with the acetyl moiety of the chitin polymer, and under acid conditions was liberated as acetic acid (Kent and Whitehouse, 1955). Alternatively, treatment of the alkali-insoluble material with chitinase yielded a soluble labeled fraction representing only 10% of the original label in the starting material. Two soluble labeled products were isolated by chromatography. Cochromatography with chitobiose and N-acetylglucosamine proved inconclusive and the products remain as yet unidentified. Matrix from dark incubations has not yet been analyzed.

DISCUSSION

The results presented here show how zooxanthellae contribute to organic productivity of a reef coral. In the light, intact corals fix considerably more $^{14}CO_2$ than those kept in the dark, due principally to photosynthesis by the zooxanthellae. As zooxanthellae fix $^{14}CO_2$ in the light, there is a progressive increase in ^{14}C content of the animal tissue until, after 24 hours, some 32–45% of the total ^{14}C in the coral is present in the animal tissue. Since freshly isolated zooxanthellae excrete photosynthate in substantial amounts in the presence of host tissue homogenate, and since the proportion of fixed ^{14}C excreted *in vitro* (up to 50%) corresponds approximately to the proportion of fixed ^{14}C accumulated by the animal tissue *in vivo*, we conclude that in the intact reef coral *Pocillopora damicornis*, zooxanthellae release a substantial proportion of their photosynthate to the animal tissue. Von Holt and von Holt (1968) reported translocation values of 40% in the coral *Scolymia* after 3 hour incubations.

The only alternative way that ^{14}C could appear in the animal tissue is through heterotrophic fixation in the light at a rate comparable to photosynthetic fixation. There is no evidence that this is the case. On the contrary, heterotrophic fixation by corals in darkness was at most 10–12% of photosynthetic fixation in the light.

The detection and measurement of translocation depends to a large extent on the efficiency of separation of animal tissue from algae. Two independent measurements used here show that the separation of *Pocillopora* constituents is relatively efficient. This is an important parameter in the analysis of translocation since relatively few algae with high specific activity could introduce significant error in analysis of animal tissue. Von Holt and von Holt (1968) reported removal of tissue from *Scolymia lacera* by "aspiration" but neither the details of the technique nor the efficiency of recovery nor the efficiency of separation of algae and animal tissues was reported. During the separation procedure host tissue homogenate continues to stimulate excretion of glycerol (Muscatine, 1967), but to no greater extent than in the intact association. Consequently some 5 minutes of *in vitro* excretion of glycerol is incorporated into each translocation experiment. If a correction for heterotrophic fixation is applied, the estimate of 35–50% translocation in a 24 hour experiment on the reef, despite its agreement with *in vitro* excretion, may actually be closer to 25–40%. This value still represents a respectable export of photosynthate by the algae, especially since apparently most free-living algae which have been studied rarely release more than 5% of their soluble photosynthate over a 24 hour test period (see Hellebust, 1965).

While we have not yet observed directly the form in which carbon is translocated in the intact coral, we suggest that some glycerol or its derivative is released by the algae and then incorporated in part into animal tissue lipid since ^{14}C-glycerol was detected in the animal tissue lipid. Release and incorporation of glycerol into lipid also occurs in *in vitro* incubations with host homogenate. But lipid is not the only animal constituent which acquires the ^{14}C label. More than half of the ^{14}C in the animal tissue was detected in the protein fraction (alcohol-TCA-insoluble). To account for this, we speculate that either some of the glycerol is metabolized to amino acids, or that other products are translocated in the intact coral but are not excreted in abundance by freshly isolated zooxanthellae. After long *in vitro* incubations with host homogenate zooxanthellae release glycolic acid, and traces of alanine and glucose (Muscatine 1967), suggesting that these might be *in vivo* translocation products. However, experiments performed by Trench (1968) show that there is a gradual change in the pattern of excretion by zooxanthellae from a variety of coelenterates after they have been removed from the host. At first, glycerol is the major constituent to be released, but three hours or more after isolation, other labeled compounds appear in greater relative proportion and glycerol excretion declines. A similar phenomenon has been observed in the lichen algae after they are separated from their fungal associates. In this case there is direct evidence that the compound moving between the algae and host fungi *in vivo* is the same as that excreted by freshly isolated algae, and that the additional compounds excreted by algae after longer *in vitro* incubations do not move *in vivo* (Smith, Muscatine and Lewis, 1969).

The observation that glucose is the major intracellular product of these zooxanthellae while a different compound, glycerol, is the major extracellular product, is consistent with observations of a similar nature in other symbiotic associations. For example, zoochlorellae from hydra and *Paramecium bursaria* manufacture and retain sucrose as their major intracellular product, but release carbohydrate as maltose (Muscatine, Karakashian, and Karakashian, 1967).

The reciprocal relationship between soluble and insoluble compounds in *Pocillopora* algae and animal tissue may be significant. Smith, Muscatine, and Lewis (1969) have discussed the maintenance by symbiotic algae of a large reserve of soluble photosynthate available for translocation. The conversion of these compounds by the host into insoluble derivatives may promote a one-way movement of photosynthate. Whereas we find that less than 50% of the ^{14}C in the animal tissue is present as soluble compounds (20% as lipid) after 24 hours, von Holt and von Holt (1968) report more than 80% of the ^{14}C in *Scolymia* animal tissue as soluble compounds (50% as lipid) after three hours incubation. We speculate that this difference is a reflection of the metabolism of different coral species, and especially different incubation times. This also serves to emphasize that the preponderance of soluble material in coral animal tissue after short incubations with $^{14}CO_2$ precludes the use of autoradiography to measure translocation. Since our incubations were much longer, our pattern of distribution of ^{14}C in animal tissue may more nearly approach a "steady state" distribution.

The skeletal matrix of *P. damicornis* consists primarily of chitin (Wainwright, 1963). The fact that a portion of this matrix is hydrolyzed with chitinase is consistent with this observation. Further studies will be required to explain the presence of the large amount of labeled alkali-soluble material associated with the decalcified skeleton. In view of their low productivity (Kanwisher and Wainwright, 1967; Halldal, 1968) it is unlikely that filamentous algae account for the labeled material. It is possible that the label is present in a small amount of protein. Protein has been shown to be associated with some native chitins (Hackman, 1960). The detection of ^{14}C in components of coral matrix may be a consequence of the relatively rapid growth rate of hermatypic corals. The ability to label the matrix may provide a tool for analysis of matrix synthesis and its relation to calcification in *Pocillopora*.

Some observations reported here on corals reveal interesting parallels with those reported for sea anemones by Trench (1969). He showed that algae from anemones and other coelenterates liberate glycerol *in vitro* and that excretion is enhanced by the presence of host tissue homogenate. He also demonstrated that in *in vivo* experiments anemones assimilate about 22–45% of the ^{14}C fixed by algae, mostly into lipid and protein.

Although the results of the present investigation provide evidence for a contribution by zooxanthellae to coral reef organic productivity, they do not demonstrate quantitatively that this represents a phenomenon of selective advantage to the hosts. This can only emerge from quantitative experiments on maintenance, growth and survival of symbiotic and algae-free coral tissues of the same species.

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SUMMARY

1. The hermatypic coral, *Pocillopora damicornis* was incubated in the laboratory and in its reef habitat with $Na_2{}^{14}CO_3$ for 1–24 hours. Controls were incubated in darkness. ^{14}C fixation in light exceeded that in darkness.

2. Fractionation of corals labeled on the reef for 24 hours revealed that 35–50% of the total ^{14}C fixed appeared in the animal tissue lipid (as ^{14}C-glycerol) and protein. From a comparison with dark controls it is concluded that photosynthetic products of zooxanthellae are translocated to host coral tissue. The skeletal organic matrix also acquires ^{14}C.

3. Zooxanthellae isolated from corals and incubated in a homogenate of host coral tissue selectively release glycerol and traces of other organic material including glucose, alanine, and glycolic acid confirming previous observations.

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NOTE ADDED IN PROOF

Mr. Stephen Young in our laboratory has analysed the distribution of ^{14}C in *P. damicornis* skeleton using a modified fractionation technique. He finds 87% as carbonate-^{14}C, 1% in protein and chitin, and 12% in an unidentified lipid. His comparative studies reveal that protein and lipid are the chief constituents of the organic matrices of most of the 14 species (4 Suborders) examined, and that chitin is the chief constiutent only in a few species, including *P. damicornis.*

Reference: *Biol. Bull.*, **137**: 524–534. (December, 1969)

INDUCTION OF OOCYTE SHEDDING AND MEIOTIC MATURATION IN *PISASTER OCHRACEUS:* KINETIC ASPECTS OF RADIAL NERVE FACTOR AND OVARIAN FACTOR INDUCED CHANGES[1]

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During the process of oogenesis in most animals the breakdown or dissolution of the nucleus (germinal vesicle) is a necessary prerequisite before meiotic maturation can occur. In starfish the induction of oocyte meiotic maturation is closely synchronized with the process of oocyte shedding from the ovary. Both events follow the exposure of ovarian tissue *in vivo* or *in vitro* to a radial nerve factor (RNF) extracted from the radial nerves (Chaet and McConnaughy, 1959; Kanatani, 1964; Chaet, 1966). Although these data indicate that radial nerve factor (RNF) acts on the gonads, it is not clear whether RNF acts directly or indirectly on the oocytes to produce shedding and meiotic maturation. Experiments by Kanatani (1964) indicated that RNF could act directly on oocytes of *Asterias amurensis*, maintained in calcium free sea water, to produce meiotic maturation. In recent studies with *Asterias forbesi* (Schuetz and Biggers, 1967; Schuetz, 1969) and *Asterina pectinifera* (Kanatani and Shirai, 1967), it was demonstrated that the RNF may act indirectly to initiate meiotic maturation. These studies showed that following *in vitro* incubation of ovarian tissue with RNF, the incubation medium, but not RNF, was capable of initiating oocyte meiotic maturation in isolated oocytes. A biologically active substance present in the incubation medium could be physically separated from the RNF. This substance has been called an ovarian factor (Schuetz and Biggers, 1967). Although the RNF and ovarian factor appeared to have different chemical properties their biological properties were remarkably similar. The so-called ovarian factor was shown to: (1) induce oocyte shedding and meiotic maturation in isolated oocytes as well as initiate meiotic maturation in oocytes retained within ligated ovarian fragments, and (2) initiate follicular cell dispersion from around isolated oocytes or oocytes retained within ovarian tissue. Such oocytes were found to be fertilizable and capable of undergoing normal development. These data suggested that this ovarian factor may be a mediator for RNF activities. Data on the time and sequence of changes which occur in oocytes and ovarian tissues following exposure to ovarian factor and RNF could provide support for this hypothesis.

The experiments reported here were conducted to elucidate some of these relationships.

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MATERIALS AND METHODS

All experiments were conducted at the Friday Harbor Laboratory, Friday Harbor, Washington. Starfish (*Pisaster ochraceus*) were collected from the intertidal zone at various times during June and July and maintained in sea tanks (10°–13° C) until utilized. All experiments were conducted under *in vitro* conditions with sea water, filtered prior to use, being utilized as the incubation medium. The ten ovaries were removed from animals and placed in large finger bowls containing sea water. The finger bowls and assay dishes used in these experiments were placed in trays on a sea table in order to maintain a relatively constant temperature. The radial nerve factor (RNF) utilized in these experiments was lyophilized material obtained from nerves of *Asterias forbesi*, prepared as previously described (Schuetz and Biggers, 1968). A stock solution of RNF was prepared by dissolving 1 mg of the dried material in 1 ml of distilled water. This solution was then kept under refrigeration (4° C). Preliminary experiments demonstrated that RNF prepared from *Asterias forbesi* was effective in stimulating oocyte shedding, meiotic maturation, and formation of ovarian factor activity when added to ovarian fragments of *Pisaster ochraceus*. This RNF material did not produce meiotic maturation in isolated oocytes. Ovarian factor utilized in the present experiments was prepared from tissue of *Pisaster ochraceus* using methods previously described, with the exception that tissues were not usually squashed prior to the addition of RNF (Schuetz, 1969). A standard preparation of ovarian factor was used in all these experiments. This substance was separated from other components of the incubation medium by gel filtration.

Biological assays

The effects of the RNF and ovarian factor were evaluated by means of the weight change in isolated ovaries or ovarian fragments and the induction of meiotic maturation in isolated oocytes (Schuetz, 1967; Schuetz and Biggers, 1968). Prior to use, ovarian responsiveness to a standard preparation of RNF was assessed. Ovarian tissues responding with 60% or greater reduction in weight were used in these experiments. Ovarian fragments were obtained by cutting ovaries into approximately equal portions. The ovarian tissues were weighed, following blotting on absorbent paper, on a Mettler P160 balance to the nearest milligram. The ovarian tissue was then placed into finger bowls or small Stender dishes containing a standard amount of filtered sea water. At various times during or at the termination of the experimental treatment, the ovarian fragments were weighed following blotting. Ovarian fragment weight loss or oocyte shedding was expressed as the ratio of the final weight to the initial weight. The ratios presented for the various groups in the figures represent the mean ratio of all fragments in a particular group.

The effects of the various biological substances or extracts on the process of meiotic maturation were assessed using oocytes isolated from pretested ovarian tissue. Mincing ovaries with scissors resulted in the release of enormous numbers of oocytes each of which was surrounded by a single layer of follicular cells. Following manual removal of the ovarian tissues present in such a mince, the remaining free oocytes with intact germinal vesicles constituted the preparation of "isolated oocytes." In all animals (> 50) studied during the summer the amount of "spontaneous" meiotic maturation in oocytes so released from ovaries was found to be less than 10%. The RNF preparation was ineffective in stimulating meiotic maturation in these oocytes. Following agitation, approximately 5000–10,000 oocytes were transferred to each assay dish containing a standard amount of filtered sea water. The test substances were then added to the dishes at varying times and for each the percentage of oocytes undergoing germinal vesicle breakdown was assessed. This was determined after agitating the dish and mixing all the oocytes. The number of oocytes with or without an intact germinal vesicle was determined within a restricted area with the aid of a dissecting microscope. A sample of at least 50 oocytes was normally examined.

Results

Time of ovarian factor appearance

In order to assess the time at which ovarian factor appeared in the incubation medium, the following experiment was performed. Four finger bowls were prepared with the following contents:

Bowl No.			
I.	1 ovary (13.7 gm)	+ 1.0 mg RNF	+ 100 ml filtered SW
II.	1 ovary (13.9 gm)		+ 100 ml filtered SW
III.		1.0 mg RNF	+ 100 ml filtered SW
IV.			100 ml filtered SW

At regular intervals following the addition of the RNF to the dishes and after agitation of the contents, a 10 ml sample of the incubation medium, free of oocytes, was removed by pipette and placed in a test tube. Shedding of oocytes became apparent approximately 70 minutes after the addition of RNF in Bowl I. The final weight of the ovary in Bowl I was 1.7 gm and in Bowl II, 13.4 gm. After all the 10 ml samples were collected, the presence of ovarian factor activity in the samples was assessed. Biological activity was determined by adding 0.5 ml from each sample collected to each of two test dishes containing free oocytes in 3.5 ml of filtered sea water. The mean results of ovarian factor activity expressed as the percentage germinal vesicle breakdown are presented in Figure 1. The data indicate that increased activity of ovarian factor was present in the media removed from the dish containing RNF and ovary together; whereas media containing only RNF or ovary were inactive in stimulating meiotic maturation. A marked change in activity of ovarian extract occurred after 100 minutes. Experiments similar to this one indicate that the time at which ovarian factor was first detected in the incubation media varied considerably and was dependent upon the amount of media tested.

RNF and ovarian factor induced shedding

The time at which oocyte shedding occurred in response to ovarian factor or to RNF were compared by recording the change in weight of ovarian fragments at varying times after addition of these two substances. Ovarian fragments were weighed and randomly placed into Stender dishes containing 4 ml of filtered sea

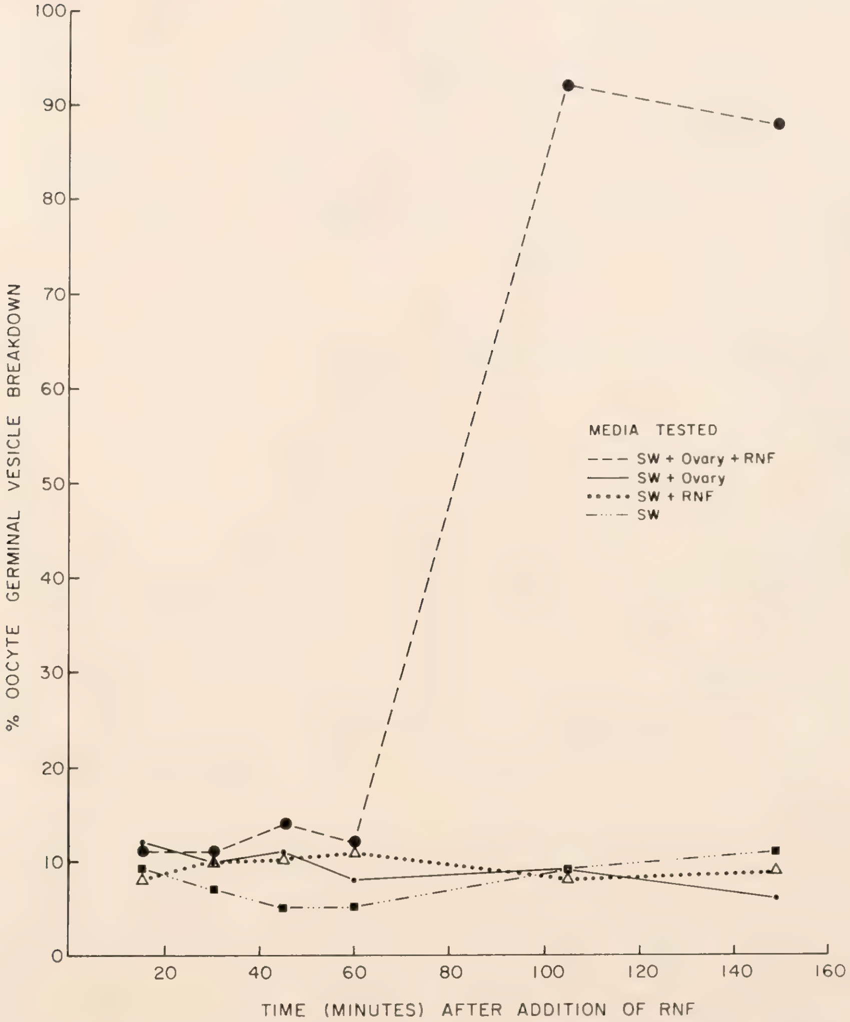

FIGURE 1. Appearance of ovarian factor activity in incubation media. Dishes of sea water were prepared with or without ovaries and with or without RNF added. Media were removed at various times and added to dishes containing isolated oocytes with intact germinal vesicles. Inducton of germinal vesicle breakdown (%) in isolated oocytes was assessed after a one hour exposure to the test media.

water. Varying amounts of ovarian factor (0.2, 0.6, 1.8 ml) or 100 μg of RNF were added to the different treatment groups. Each treatment group consisted of 5 fragments each in an individual dish. At varying times after the addition of these test substances, each fragment was removed, blotted, weighed, and then returned to the same dish from which it was taken. The sequential changes in weight of the variously treated fragments are presented in Figure 2. The data are presented as the mean ratio of the final weight to initial weight, but the final

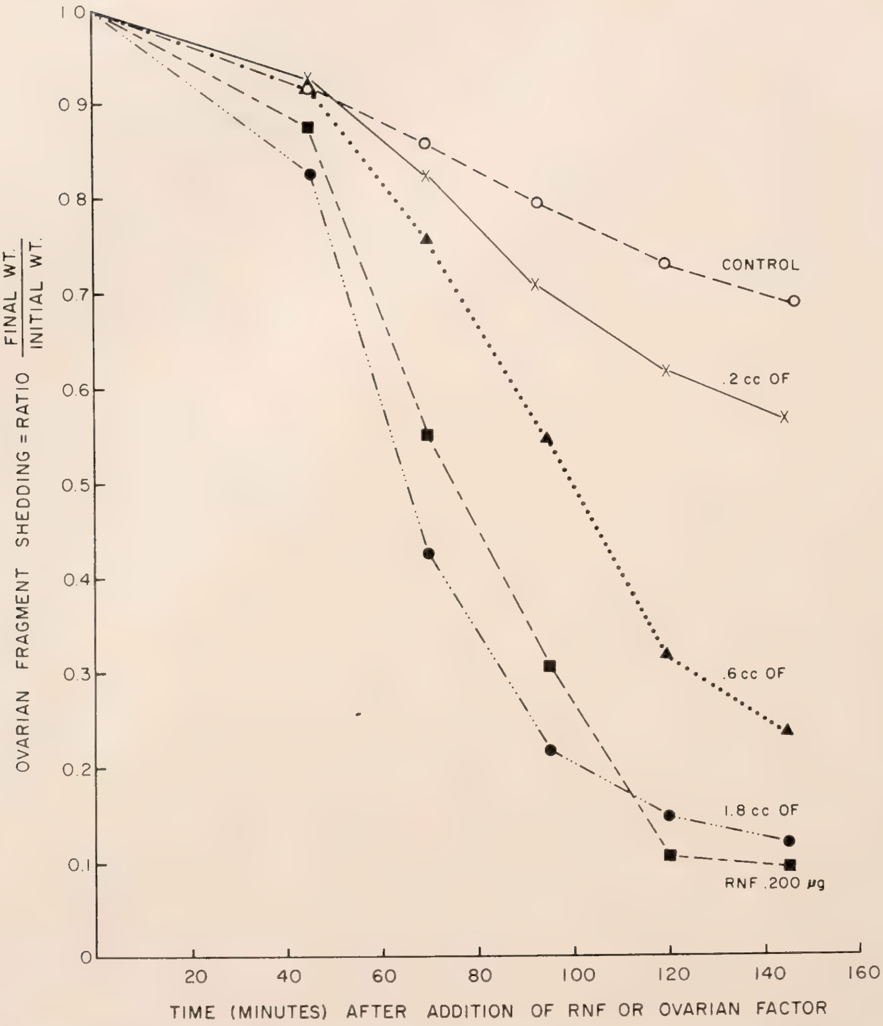

FIGURE 2. Ovarian fragment shedding—Effect of ovarian factor and RNF. Groups of five ovarian fragments were exposed to RNF or various concentrations of ovarian factor. The data represent the sequential change in weight of these fragments.

weight is in reality the weight of the fragment as measured at the designated times after the addition of RNF. These data indicate that little shedding occurred before 50 minutes after the test materials were added. The extent of shedding in response to ovarian factor was markedly dependent upon the dose added. Shedding occurred in a remarkably similar fashion in those fragments exposed either to the highest dose of ovarian factor or to RNF. Repetition of this experiment demonstrated the same type of finding; however, the time at which shedding was initiated varied in the different animals.

Effect of RNF exposure time on oocyte shedding

It is not known whether, or for how long, RNF is required to be in the presence of ovarian tissue in order for shedding to occur. This was tested by exposing groups of ovarian fragments to RNF for varying periods of time, washing them and returning them to fresh media containing no test material. Randomized groups of ovarian fragments were exposed to 100 μg of RNF in 15 ml of filtered sea water. One control group did not receive any RNF and one

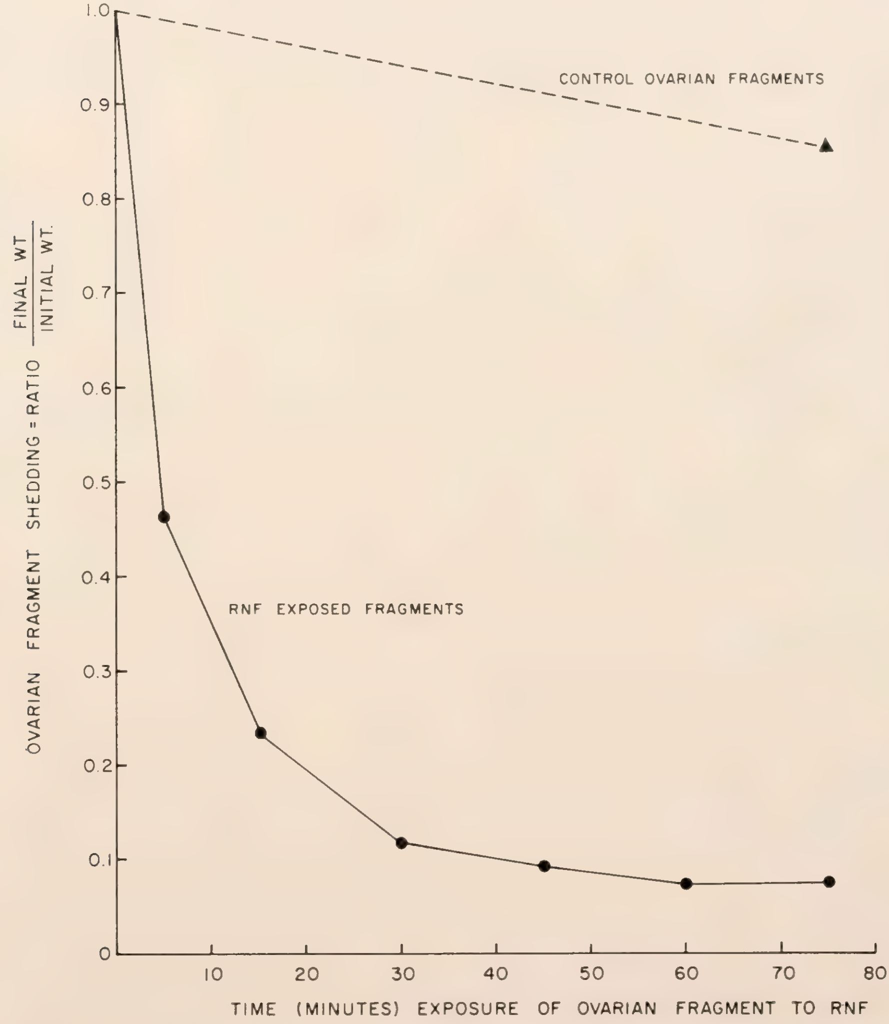

FIGURE 3. Ovarian fragment shedding—Effect of time of exposure to RNF. Groups of five ovarian fragments were exposed to RNF for various times (min). The tissues were washed and transferred to fresh media without RNF. Each dot represents the mean ratio of the five fragments.

group was exposed to RNF during the entire experimental period. After the designated period of exposure to RNF the fragments were removed, blotted and transferred through 3 washes (in a 6 minute period), each consisting of 300 ml of fresh sea water. Following washing fragments were placed in fresh sea water (15 ml). The final ovarian weight of all fragments was recorded 120 minutes after the fragments were originally exposed to the RNF. The results of this experiment are presented in Figure 3. A 5 minute exposure to RNF was sufficient to induce a 50% reduction in ovarian weight, whereas, a 30 minute exposure insured maximal shedding within the two hour test period. Shedding did not occur in control fragments incubated in the continual presence of RNF until after 60 minutes. Shedding appeared to occur sooner in those fragments exposed to RNF, and subsequently washed prior to being returned to RNF-free media, than in those exposed continuously to RNF.

Oocyte response to ovarian factor

The time at which germinal vesicle breakdown occurs in oocytes following exposure to ovarian factor and the length of time oocytes need to be exposed to ovarian factor are questions of importance. Free oocytes (5000–10,000) in 0.5 ml were added to 5 ml of filtered sea water and then 1 ml of the standard preparation of ovarian factor was added. Controls consisted of oocytes not exposed to ovarian factor or to RNF. Oocytes were incubated in the continual presence of ovarian factor or were exposed to ovarian factor for varying times and then transferred to fresh sea water containing no ovarian factor. Both parts of this experiment were performed using oocytes obtained from the same animal. The results of both aspects of this experiment are presented in Table I. In the first instance, 7 dishes containing oocytes were exposed to ovarian factor. At varying intervals after the addition of the ovarian factor, the percentage of oocytes with intact

TABLE I

Time of germinal vesicle breakdown in oocytes following exposure to ovarian factor (1 ml) and the effect of time of exposure of oocytes to ovarian factor (1 ml) germinal vesicle breakdown

Part I		Part II	
Time (min) after ovarian factor added to oocytes	% oocyte GVBD	Time (min) oocytes exposure to ovarian factor	% oocyte GVBD
0	6%	0	5%
5	7%	5	5%
10	9%	10	15%
15	10%	15	98%
20	12%	20	98%
25	25%	25	97%
30	85%	30	98%
35	98%	35	99%
Terminal control			
RNF (100 μg) treated oocytes	5%		7%

germinal vesicles in one dish was immediately recorded. The entire process of oocyte germinal vesicle breakdown occurred within a 3–5 minute period and was synchronous in all the oocytes released from a particular ovary. Germinal vesicle breakdown occurred in 98% of the oocytes 35 minutes after the addition of the ovarian factor.

For the second part of this experiment, 8 dishes of oocytes were exposed to ovarian factor and at subsequent 5 minute intervals one dish was flooded in turn with fresh sea water. This medium was poured off and the remaining oocytes were immediately transferred to a dish containing 100 ml of fresh sea water. Addition of 1 ml of ovarian factor to oocytes in 100 ml of sea water was ineffective in stimulating meiotic maturation. One hour after the initial exposure of oocytes to the ovarian factor, the percentage of germinal vesicle breakdown in all dishes was determined. Exposures of 5–10 minutes were insufficient to produce germinal vesicle breakdown, whereas, longer periods of time produced essentially 100% germinal vesicle breakdown. This breakdown occurred at essentially the same time as in those oocytes incubated in the continual presence of ovarian factor.

The time at which germinal vesicle breakdown occurred in isolated oocytes was also compared to the time of shedding from ovarian fragments exposed to RNF or ovarian factor. In each case these comparisons were made using free oocytes and ovarian fragments obtained from the same animal. These experiments, using tissue and oocytes from different animals, revealed in all cases that oocyte germinal vesicle breakdown induced with ovarian factor preceded shedding induced with either ovarian factor or RNF.

Discussion

Previous experiments have indicated that changes in the starfish ovary previously attributed to the actions of radial nerve factor (RNF) may also be produced by a possible intermediary substance called an ovarian factor (Schuetz and Biggers, 1967; Schuetz, 1969) or a meiosis-inducing substance (Kanatani and Shirai, 1967). In general, data presented here support this contention. The physiological changes studied were the processes of oocyte shedding and oocyte germinal vesicle breakdown.

Shedding of oocytes, following exposure of ovarian fragments to either RNF or ovarian factor, was remarkably similar in character and duration. The process of induced oocyte shedding, as reflected by the changes in the weight of the ovarian fragments, was divided into distinct phases (Fig. 2). Subsequent to the addition of RNF or ovarian factor, an initial lag phase occurred during which the weight of the ovarian fragment showed little change. In the data presented here, the duration of the lag phase was approximately 50 minutes. This initial phase was followed by a continuous decrease in the weight of ovarian fragments over a 50 minute period. In fragments exposed to ovarian factor the rate of weight change was markedly dependent upon the dose of ovarian factor present in the assay dishes. The rate and extent of weight loss in the ovarian fragment following exposure to either the RNF or the highest dose of ovarian factor were indistinguishable.

Previous investigators have described the presence of a lag phase for RNF-induced shedding (Chaet and Musick, 1960); however, the duration of the lag phase appears to vary considerably with the species and animal tested. Ovaries of *Pisaster ochraceus* exposed to RNF were consistently found to have a longer lag period than similarly treated ovaries of *Asterias forbesi* (Schuetz unpublished). Whether this is a true species difference or the result of the water temperature differences on the east and west coasts is not clear.

Although oocyte shedding and meiotic maturation resulted when ovarian fragments were exposed to RNF, meiotic maturation in isolated oocytes occurred only when exposed to ovarian factor (Fig. 2), and took approximately 30 minutes for completion (Table I). Thus, ovarian factor acted more rapidly in producing germinal vesicle breakdown in isolated oocytes (Table I) than in producing oocyte shedding (Fig. 2). This was true even when similar concentrations of ovarian factor were present in assay dishes containing oocytes and ovarian fragments. Thus, these data suggest that meiotic maturation, which occurs following ovarian tissue exposure to RNF, could be explained by an intermediate step since the time required for the ovarian substance to produce its effects was less than that required for the RNF. The question arises, however, of why its effect on the germinal vesicle in isolated oocytes (Table I) is more rapid than its effect on shedding of ovarian fragments (Fig. 2). Previous data demonstrated that the ovarian factor (Schuetz, 1969) and RNF (Kanatani, 1964; Schuetz, 1969) can act through the ovarian wall of ligated ovarian fragments to initiate meiotic maturation. A possible explanation is that the ovarian tissues are differentially permeable to one of these substances. Alternatively, if the RNF is producing or releasing ovarian factor from ovarian tissue, this ovarian factor will then be in closer proximity to the enclosed oocytes than the ovarian factor present in the incubation media.

In what cells or tissues the ovarian factor is found and whether it is released or synthesized in response to RNF is not at present clear. The time relationships described here (Fig. 3 Table I) indicate that RNF "activation" of ovarian factor is a relatively rapid process. It is not clear how the ovarian factor produced the changes in the ovarian fragments leading to oocyte shedding. Previous studies have indicated that disruption of the follicular cells from around oocytes may be a necessary prerequisite for shedding (Kanatani, 1964). The muscle tissues of the ovarian wall also appear to be important for the shedding response (Schuetz and Biggers, 1969; Kanatani, 1964; Macklenberg and Chaet, 1964). How these tissues and inductor substances interact to produce these changes is not yet clear. The induction of meiotic maturation in oocytes, however, appears to result from the direct action of the ovarian factor. The inability of the RNF to act directly on the isolated oocytes to produce germinal vesicle breakdown suggests that the oocyte is not itself the source of the ovarian factor. The possibility does exist, however, that the RNF, although not acting directly on isolated oocytes, could act on the non-shed oocytes causing them to release the ovarian factor. Kanatani *et al.* (1969) recently presented experimental evidence indicating that the meiosis inducing substance, which is comparable to the ovarian factor discussed here, is 1-methyladenine. The elucidation of the physiological and biochemical role of this substance should greatly enhance our understanding of the process of oogenesis.

The author acknowledges the many helpful suggestions of, and the necessary equipment provided during the course of this investigation by, Dr. Robert Fernald, Director of the Friday Harbor Laboratories.

Summary

The addition of a radial nerve factor (RNF) to starfish ovarian tissue *in vitro* results in the release into the incubation media of a substance (ovarian factor) with different chemical properties but many biological properties similar to those of the RNF. A series of experiments were conducted to assess and compare the time course during which these substances initiate their biological effects. The effects of these substances on the process of oocyte shedding and germinal vesicle breakdown were assessed under *in vitro* conditions.

1. Within approximately a 1 hour period following the addition of RNF, ovarian factor activity was found in the incubation media. Activity in some instances was found prior to induction of shedding.

2. Oocyte shedding induced by the addition of both RNF and ovarian factor occurred folowing a lag period of approximately 1 hour. The rate of oocyte shedding in response to RNF and the highest dose of ovarian factor were indistinguishable.

3. Free oocytes exhibited germinal vesicle breakdown in response to ovarian factor but not to RNF. Germinal vesicle breakdown in response to ovarian factor occurred within 30 minutes of the addition of ovarian factor to free oocytes. Germinal vesicle breakdown in free oocytes occurred prior to either ovarian factor or RNF induced shedding from ovarian fragments.

4. The continual presence of RNF in the incubation media was not required for the induction of shedding from ovarian fragments.

5. Free oocytes did not require the continual presence of the ovarian factor for germinal vesicle breakdown to occur. An exposure time of approximately 15 minutes insured meiotic maturation in free oocytes.

These data support the hypothesis that the ovarian factor is a normal intermediary substance in RNF-induced shedding and in oocyte germinal vesicle breakdown or meiotic maturation.

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VISUAL FORM DISCRIMINATION BY ECHOLOCATING BATS

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Visual information is generally thought to be of little importance to echolocating bats, many of which exhibit a great diversity in their diets and behavior. Although all apparently rely heavily on acoustic orientation, one might expect that non-auditory senses such as vision and olfaction could provide valuable supplements to audition in the performance of certain activities. Visual pattern perception could also aid in homing and long distance navigation if celestial bodies or landmarks beyond the range of echolocation can be seen.

A number of tropical species possess relatively prominent eyes which may aid them in finding fruit, nectar or in the case of vampires, large mammals. However, previous experiments have failed to demonstrate pattern vision. Eisentraut (1950) succeeded in conditioning *Eptesicus serotinus* and *Plecotus auritus* to distinguish white from black cards or doorways by rewarding proper choices with food but was unsuccessful in an attempt to train them to discriminate between a cross and a circle. Curtis (1952) similarly conditioned *Myotis lucifugus* and *Eptesicus fuscus* to search for food at the light, rather than dark end of a test chamber, but these bats did not distinguish between stripes subtending one and eight degrees. Light-dark discrimination has also been shown for *Myotis sodalis* (Davis and Barbour, 1965). The results of these and other experiments supported the supposition that the Microchiropteran eye was probably able to do little more than distinguish light from dark. Suthers (1966; in press) however, obtained optomotor responses to moving stripes suggesting minimum separable angles of less than one degree in some species. Bradbury and Nottebohm (in press) and Chase and Suthers (1969) demonstrated that deafened *Carollia perspicillata*, *Phyllostomus hastatus* and *Myotis lucifugus* can visually detect and avoid obstacles placed in their flight path. Vision has, in fact, recently been implicated as an aid to orientation during homing (Williams and Williams, 1967).

In this paper we present evidence that at least two species of Microchiroptera are able to distinguish visually between simple stationary patterns. Of these, *Anoura geoffroyi* (Gray) is nectivorous and *Carollia perspicillata* (Linnaeus) is frugivorous. Both are nocturnally active and emit ultrasonic cries which they use in echolocation.

METHODS

A hungry bat was conditioned to seek food at one member of a pair of equal area patterns presented simultaneously as silhouettes against a diffusely trans-illuminated background. One pattern was always associated with edible food (serving as positive reinforcement), whereas the other was associated with a

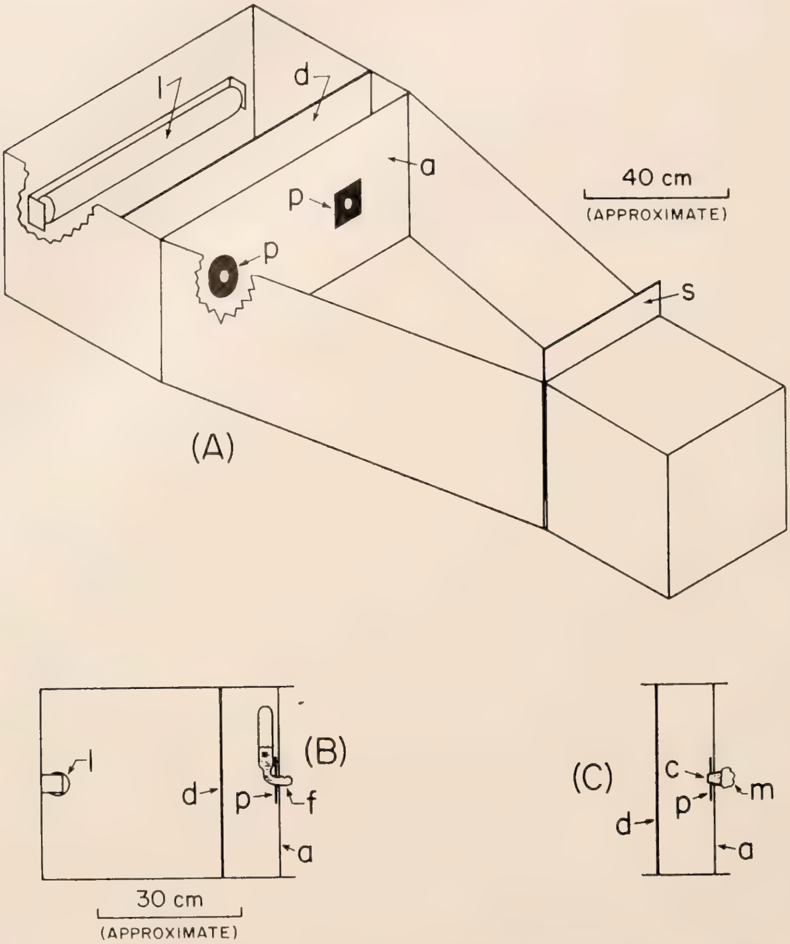

FIGURE 1. A. Conditioning apparatus used for *Anoura geoffroyi* and *Carollia perspicillata*. B. Detail (side view) showing dispenser with sucrose or control solution as presented to *A. geoffroyi*. C. Detail (side view) showing manner in which melon ball was presented to *C. perspicillata*. a, clear acrylic plastic sheet; c, cork with peg holding melon ball; d, translucent diffusing plate of frosted glass; f, glass fluid dispenser used with *A. geoffroyi*; l, fluorescent or incandescent light bulb; m, melon ball; p, patterns; s, starting point for bat at beginning of each trial.

distasteful aqueous quinine solution or quinine-treated food (serving as negative reinforcement) or with no food.

Anoura and *Carollia* were tested in a plywood box with an open top covered by wire mesh (Fig. 1a). Both of these genera are capable of stationary, hovering flight and flew easily within this chamber. The *Carollia* to be tested was isolated behind a removable, opaque partition 105 cm from the test patterns. On the other side of this partition the chamber gradually widened toward a 78 × 30 cm sheet of clear acrylic plastic. The test patterns were constructed of heavy, opaque, black poster cardboard and mounted on the back of this clear plastic sheet with their centers 40.5 cm apart. A frosted glass diffuser panel of similar dimensions was mounted 10 cm behind the clear plastic, parallel to it. A 20 watt white fluorescent bulb centered 30 cm behind this diffuser provided an illuminance of about 50 apparent foot-candles on the plastic surrounding the patterns. Positive or negative reinforcement was provided at the center of each pattern through an 18 mm diameter hole in the plastic and cardboard. The relative positions of the two patterns were interchanged semi-randomly between trials. The transparent plastic sheet between the bat and the cardboard pattern prevented echolocation of the latter.

At the beginning of each trial the partition isolating the bat from the patterns was raised and the animal was allowed to fly toward the patterns. The bat was considered to have made a choice when it touched the edible or quinine-treated food, or their dispensers. A trial was termed a success if this first contact occurred at the positively rewarded pattern. Contacts at either pattern were usually accompanied by tasting or eating. Sometimes the bat flew on a nearly straight line directly to one of the patterns as soon as the partition was raised. Often several flights were made near one or both patterns before the animal attempted to eat at one of them. When a high per cent of correct choices indicated conditioning had taken place, control trials were randomly interspersed during which identically treated edible food or pure water was placed at both patterns in order to establish that choices were based on visual rather than possible olfactory clues. Prior to each olfactory control trial, the surface of the plastic sheet was thoroughly washed to remove odors.

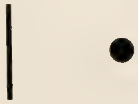

FIGURE 2. Pattern pair consisting of disc and equal area rectangle. *Anoura geoffroyi* was conditioned to seek food at the disc.

A test chamber of slightly different dimensions was used during the disc *vs.* rectangle experiments with *Anoura* (Fig. 2). The patterns were spaced 30 cm apart on a 60 × 30 cm sheet of clear acrylic plastic which was trans-illuminated by two 15 watt incandescent bulbs behind a sheet of frosted glass. The illuminance at the surface of the clear plastic was about 200 apparent foot-candles (*i.e.*, assuming a human phototopic luminosity curve for the bat). The reward consisted of an approximately 1 molar aqueous sucrose solution. Negative reinforcement was provided by an aqueous quinine solution. Each solution was presented in a glass water dispenser mounted behind the pattern with its delivery spout extending about 2 cm into the test chamber through a hole at the center of the pattern. Several dispensers were used and occasionally interchanged to prevent the bat from associating any minor peculiarity in the shape of a particular feeder with the sucrose or quinine. *Anoura* fed by hovering in front of the dispenser and extending its long tongue into the fluid (Fig. 3).

Another *Anoura* was later tested on triangles, squares, and discs in a similar box (Fig. 4). The opaque partition in this apparatus was 137 cm from the test patterns which were mounted 40.5 cm apart on a 78 × 53 cm sheet of clear plastic.

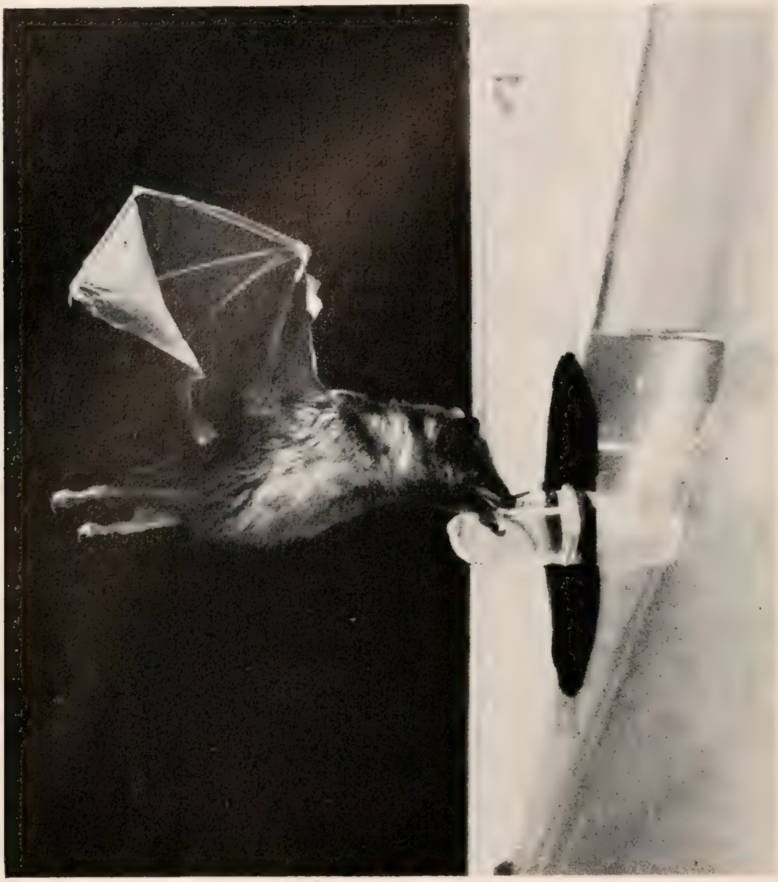

FIGURE 3. *Anoura geoffroyi* hovering in front of pattern while drinking from feeder. Note long tongue extending into sucrose solution.

A 20 watt white fluorescent bulb was centered 42 cm behind the frosted glass diffuser. Water dispensers protruded through holes in the clear plastic immediately below each pattern rather than at its center.

During experiments with *Carollia,* each glass feeder was replaced by a melon ball (*ca.* 2.5 cm in diameter) attached to a cork which fitted into the hole at the center of each pattern. Commercially available frozen melon balls were thawed and washed in water to remove excess packing syrup. Some were then soaked in an aqueous sucrose solution to be used as positive reinforcement; others were soaked in an aqueous quinine-sodium chloride solution to serve as negative reinforcement. Since the melon balls were somewhat irregular in shape, they were periodically replaced during each series of trials to prevent the bat from associating a particular one with reward or punishment. Olfactory controls were accomplished by occasionally placing sucrose treated melon balls at both patterns after washing the surface of the plastic.

Results

Anoura geoffroyi

Our first experiments were conducted with this nectivorous bat. The animal was allowed to become accustomed to the test chamber by feeding in it for a few days prior to the presentation of patterns. The first pattern pair consisted of a postively rewarded ring and a negatively reinforced cross with the sucrose and quinine dispensers situated a few centimeters in front of the patterns. This distance was apparently enough to make it difficult for the bat to associate the feeder with the pattern. During nine days of experiments the animal's score occasionally approached statistical significance but fluctuated too much from day to day for a clear result to be obtained. The apparatus was then redesigned so that a feeder projected directly out of the center of each pattern as described above.

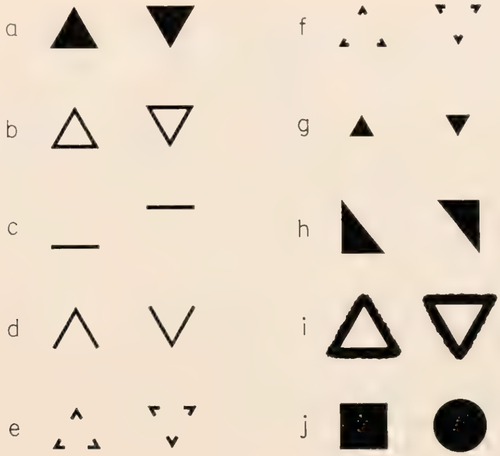

FIGURE 4. Pairs of patterns used in analysis of discrimination between erect and inverted triangles by *Anoura geoffroyi*. See text.

Disc vs rectangle Simultaneously with the rearrangement of feeder position the test patterns were changed to a solid disc 73 mm in diameter and a long, narrow vertical rectangle (15 × 280 mm) of equal area (Fig. 2). Sucrose was associated with the disc, quinine with the rectangle. Conditioning took place rapidly and after 51 trials in two days the animal consistently made more than 70 per cent correct choices. During the next three successive days, this specimen of *Anoura* chose the sucrose solution in 83.1 per cent of 89 trials ($P < 0.01$) and in 100 per cent of eight olfactory control trials ($0.01 < P < 0.02$) in which only water was present at both patterns. The bat's performance improved each day until on the final day of testing it scored 92.9 per cent correct choices in 28 trials. The rapidity with which this discrimination was learned may be misleading, however, since there could have been some transfer from the ring used in the initial tests to the disc.

Analysis of discrimination between erect and inverted triangles We subsequently tested a second specimen of *Anoura* with a view to determining what aspects of simple patterns are utilized in their visual discrimination and also in

TABLE I

Visual form discrimination and transfer of learning by Anoura geoffroyi

Pattern pair	Number of trials	Per cent successes	Value of *P*
a	92	83.7	<0.01
b	17	88.2	<0.01
c	15	93.3	<0.01
d	15	53.3	~1.0
e	15	86.7	<0.01
f	20	70.0	<0.20
g	32	68.7	<0.10
h	15	86.7	<0.01
i	10	50.0	<0.80
j	15	93.4	<0.01

order to obtain a more direct comparison of the visual form discrimination of a bat with that of some other non-ecolocating mammal. This comparison was facilitated by using patterns similar to those which Lashley (1938) presented to rats.

An *Anoura* was trained to visually distinguish between a pair of identical equilateral triangles measuring 10 cm on a side. One triangle was erect, the other inverted (Fig. 4a; Table Ia). Sucrose solution was associated with the erect triangle and a quinine solution with the inverted triangle. In all experiments with this bat, the position of the patterns was determined from Gellerman's (1933) table of trial orders to avoid scores due to position habits, simple alternation, and double alternation. The first five days of testing (90 trials) during which the bat was being conditioned are not included in Table I. Olfactory controls using sucrose solution at both patterns were performed with every pair of patterns and are included in the trials of Table I. When the value of *P* for the non-olfactory control trials was less than 0.05, the value for olfactory control trials was also less than 0.05. In no case was there evidence that choices were being made on the basis of olfactory stimuli.

After *Anoura* was conditioned to seek food at the solid, erect, equilateral triangle we confronted it with other pattern pairs selected in an effort to determine upon what specific aspects of the triangles, pattern discrimination depended. If the conditioning transferred to the new patterns, it could be assumed they retained a distinguishing characteristic of the original pair of triangles. Such transfer test patterns were presented to the bat on only one night for from 10 to 32 successive trials—nearly all of which were olfactory controls. On the night following each such test for transfer of conditioning the bat was retested for about 10 trials on the original equilateral triangles.

From Table I and Figure 4 it can be seen that this *Anoura* which was trained on solid equilateral triangles (a) transferred its conditioning to the complete outlines (b) of these triangles and to the baseline (c) of each triangle presented above, but not to the two sides without a base (d). When the corners of the triangles were presented, *Anoura* was able to choose correctly if the side segment forming each corner extended 1.5 cm from the inside angle (e) but not when this length was reduced to 1 cm (f). Small solid equilateral triangles measuring 5 cm on a side (g) were distinguished on only 68.7 per cent of the trials. Conditioning to equilateral triangles transferred to right triangles (base 9 cm, height 11 cm) (h). The bat's score fell to a chance level when the contrast was reversed so that translucent equilateral triangles 10 cm on a side appeared in a black surrounding (i). Initial trials prior to conditioning are omitted from Table Ia but are included in the learning curve in Figure 5.

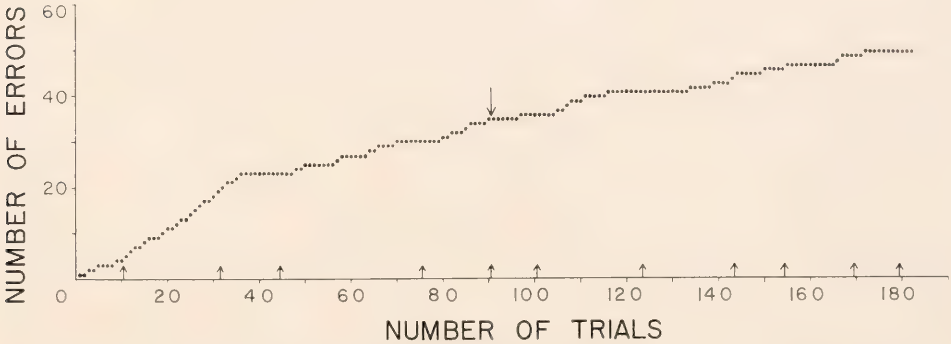

FIGURE 5. Learning curve of *Anoura geoffroyi* for erect and inverted equilateral triangles 10 cm on each side. Upward pointing arrows along abscissa indicate successive days of testing. Large arrow touching curve between fifth and sixth day indicates beginning of trials included in Table I. Learning may be considered to have occurred as early as the third day, depending on the criterion used.

These data suggest that *Anoura* depended almost exclusively on the baseline of the pattern for making its choices. Failure to differentiate between members of pair (f) may be explained as due to the less complete baseline in comparison to (e) or possibly to an excessive demand upon the animal's visual acuity. Failure in the case of pair (g) is difficult to understand unless it is due to the shortness of the baseline relative to that of the training patterns.

Immediately following the preceding experiments involving triangles the same bat was confronted with a 10 × 10 cm square above the sucrose solution and an equal area (11.2 cm diameter) disc (j) above the quinine solution. The conditioning to the equilateral triangles also transferred to this pair of patterns. The square was selected in 93.4 per cent of 15 trials on the first night of its presentation. The only error was made on one of eleven olfactory control trials included in the above total ($P < 0.01$ for all trials; $0.01 < P < 0.02$ for olfactory controls). The base of the square was in the same position and orientation as the base of the erect triangle and must have been the basis of its selection by the bat. Since the top of the square similarly resembled the base of the inverted triangle, the success of the bat suggests that it no longer heeded the negative pattern and relied exclusively on the presence of a positive pattern baseline. This test was performed after 28 days of testing on the other patterns in Figure 4. It is interesting to note that this reliance on the baseline of the positive triangle apparently developed to the exclusion of other aspects of the pattern pair only after many days of conditioning. Thus when the same bat was presented with the same square and circle, each with sucrose solution, after 18 days of conditioning there was no transfer, the correct choice occurring on only 42.9 per cent of 14 trials.

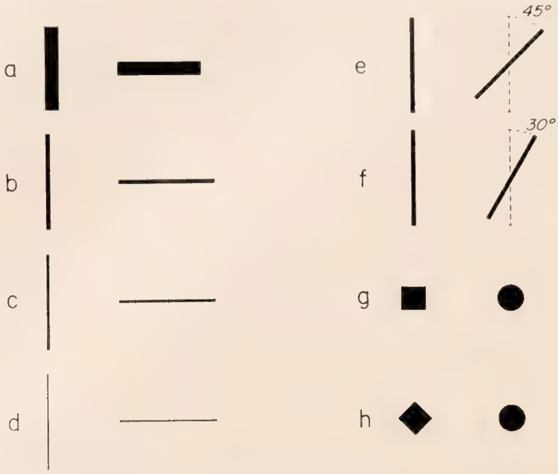

FIGURE 6. Pairs of equal area patterns presented to *Carollia perspicillata*. Scale is approximate. See text.

Carollia perspicillata

One *C. perspicillata* learned to distinguish between two identical rectangular stripes—a vertical one associated with food and a horizontal one associated with quinine (Fig. 6). During the first series of experiments 40 × 243 mm rectangles (a) were used. In three successive experiments the length of these rectangles was increased to 280 mm and their width was reduced to 14 (b), 7 (c), and finally 3.5 mm (d), respectively. Trials prior to conditioning on the 40 mm wide rectangles are not included in line (a) of Table II. Once the bat learned to distinguish between the vertical and horizontal member of this pair the animal maintained its performance when presented with 14 or 7 mm wide rectangles. Specimens of *Carollia* scored about 90 per cent correct choices with these three wider pairs of rectangles. The slight improvement in performance with 14 and 7 mm wide rectangles is probably simply the result of additional training, since there was good transfer of learning between these pairs. One may alternatively

TABLE II

Visual form discrimination by Carollia perspicillata

Pattern pair	Number of trials	Per cent successes	Value of P
a	95	89.5	<0.01
b	65	92.3	<0.01
c	60	93.3	<0.01
d	85	52.9	$0.50>0.70$
e	108	75.0	<0.01
f	298	68.5	<0.01
g	75	80.0	<0.01
h	40	90.0	<0.01

hypothesize that the narrower the rectangles the more strongly was their contrasting orientation emphasized and that this facilitated correct choices by the bat. When the width of the rectangle was further reduced to 3.5 mm, the score abruptly dropped to about the 50 per cent predicted by chance selection. When trials with wider rectangles were inserted between those with these narrowest rectangles, the bat readily made the correct choices—indicating the poor performance on the 3.5 mm wide rectangles was not due to a lack of motivation, but probably instead to excessive demands on the bat's acuity or perhaps simply to a decreased conspicuousness of patterns that remained visible.

The same *Carollia* was subsequently presented with a pair of 14 × 280 mm rectangles in which the postively reinforced member of the pair was oriented vertically as before, but the long axis of the negatively reinforced rectangle formed an angle of 45 (and, in later experiments, 30) degrees with the vertical (Fig. 6e and f; Table II). Although the bat's performance progressively diminished as the long axis of the negative rectangle was rotated toward the vertical orientation of the rewarded pattern, even when this difference was reduced from 90 to 30 degrees, the *Carollia* scored nearly 70 per cent correct choices.

A second *Carollia* demonstrated an impressive ability to distinguish between a solid square, 7 cm on each side, and an equal area solid disc with a diameter of 7.8 cm (Fig. 6g and Table II). When this square was rotated 45 degrees so that it appeared to stand on one corner (Fig. 6h) the bat continued to make the distinction without having to relearn the problem. This indicates that *Carollia* must indeed discriminate between these patterns on the basis of their shapes—the corners and straight edges of the square contrasted to the curved edge of the disc—and not on the basis of small spatial differences in illumination which must exist even though the average luminous flux is the same around each pattern.

Discussion

Limited data on the visual acuity of twelve echolocating bats was obtained by Suthers (1966 and in press) on the basis of optomotor responses to moving stripes. *Anoura geoffroyi* and *Carollia perspicillata* responded to stripes subtending 0.7 and 0.5 degrees, respectively.

The present experiments do not provide data on visual acuity since there was no fixed distance at which the bat was forced to make a decision. Attempts to force *Anoura* to make a choice at a predetermined distance from the patterns were unsuccessful. Neither could a consistent choice point be determined by tracing the bat's flight path towards the patterns. Although conditioned animals often seemed to fly directly to the correct pattern, the point at which their flight deviated to the left or right was difficult to determine since the initial portions of even direct flights were very similar. At other times the bat would approach to within a few centimeters of first one pattern and then the other before choosing. The narrow stripes of Figure 6d may have placed excessive demands on the acuity of *Carollia*. However, the bats should have been able to resolve the major features of the other patterns used.

The subordinate role of vision as a telereceptive sense is indicated by the fact that our experimental animals resorted to visual stimuli only after failing to obtain a reliable indication of the position of the food through place learning, echolocative or olfactory stimuli. Similar behavior has been noted in other "nonvisual" animals and it is interesting to compare the visual form discrimination of bats with that of a non-ecoholcating nocturnal mammal. One of the nocturnal mammals most thoroughly studied in this respect is the rat. Our experiments testing the ability of *Anoura* to transfer discrimination from triangles to other patterns are similar to some of those conducted by Lashley (1930, 1938) and Munn (1930) with pigmented rats. Visual pattern discrimination experiments using the rat are summarized by Munn (1950).

Rats are similar to bats in having nocturnally adapted eyes with all or nearly all rod retinae lacking a fovea. Rats which Lashley and Munn trained to discriminate between erect and inverted equilateral triangles transferred to triangular outlines. Most rats transferred to two sides, some transferred to the bases alone, and about one-tenth of the rats tested responded to the lower angles. Large changes in the size of the equilateral triangles or the substitution of right triangles did not affect the performance of the rat (Fields, 1932). Reversal of contrast, however, destroyed the rat's ability to discriminate. Rats are apparently unable to distinguish between equal area squares and circles. Allowing for some individual variations, it appears that *Anoura geoffroyi* is capable of form discrimination at least comparable to that of pigmented rats. In contrast to rats, all of our bats were able to distinguish between squares and equal area circles, suggesting that their form perception may be in some respects superior to that of rats.

Tests showing transfer of form discrimination raise the question as to whether or not the animal possesses an abstract concept of form such as triangularity (see Fields, 1932). In the absence of such a concept, form discrimination may depend on a particular retinal distribution of light or the relationship of a pattern to its surroundings. The fact that a specimen of *Anoura* transferred from equilateral triangles to three corners and to right triangles suggests it may have had some awareness of triangularity. This interpretation is cast in doubt, however, by the animal's transfer to horizontal rectangles representing the base lines of triangles. The successful transfer by *Carollia* to a rotated square (Fig. 6h) could be interpreted to imply some degree of abstraction of the concept of squareness or circularity by this bat but further careful experiments are needed. Such abstractions, as pointed out by Lashley (1938), should probably be considered as evolutionary refinements of the fundamental capacity for generalization rather than as unique or sudden evolutionary advances.

The ability for form discrimination has been studied in a number of other animals, but the variety of patterns presented and experimental techniques employed make a meaningful comparison difficult. Karn and Munn (1932), however, conditioned two mongrel dogs to distinguish between erect and inverted equilateral triangles and found that they transferred not only to complete outlines, the three corners, and the baseline, but also to the two sides without a baseline. Since *Anoura* did not treat this latter pattern as equivalent to the original triangle, bats and dogs may differ in the means by which they perceive these forms. Sutherland (1957) has hypothesized a mechanism of form discrimination based on extensive experiments with the octopus (*Octopus vulgaris*). He suggests that the octopus visual system may distinguish between forms on the basis of their vertical and horizontal extents or projections. The pattern pairs which we presented to our bats did not include critical experiments to test this hypothesis for these animals. The ability of *Anoura* to distinguish between identical horizontal bars representing the bases of triangles (Fig. 4c) and its inability to discriminate between the sides of erect and inverted triangles (Fig. 4d), however, suggests that the Microchiropteran visual system must utilize more than these two aspects of forms in making a discrimination. Different mechanisms of form discrimination in these animals would not be surprising considering the phylogenetic remoteness of bats from cephalopod molluscs.

Why should a bat which has evolved a sophisticated capacity for echolocation also maintain a comparatively good visual system? The answer may lie in the relatively limited range of effective echolocation due to atmospheric attenuation and spreading losses of sound. Visual recognition of large objects or landscapes beyond the range of echolocation may assist echolocating bats in orienting themselves on long distance flights to feeding trees or sites. *Phyllostomus hastatus* apparently use visual orientation to home from distances greater than about 10 km (Williams, Williams and Griffin, 1966; Williams and Williams, 1967). Chase and Suthers (1969) and Bradbury and Nottebohm (in press) obtained evidence that *Carollia, Phyllostomus,* and *Myotis* are able to visually detect and avoid certain stationary obstacles during flight. Although Microchiropteran eyes are some what hypermetropic, this refractive error is probably insignificant compared to the total power of the eye and their great depth of field probably compensates for the absence of a well developed accommodative mechanism (Suthers and Wallis, in preparation). Vision is certainly a functional, if secondary sense of echolocating bats. Its importance varies with the species' behavior and ecology.

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SUMMARY

Two species of echolocating Microchiroptera were behaviorally conditioned to visually discriminate between various pairs of simple, equal area patterns presented simultaneously as silhouettes against a diffusely trans-illuminated background. These were *Carollia perspicillata* (frugivouous) and *Anoura geoffroyi* (nectivorous). Data suggest that visual form discrimination in bats such as *Anoura geoffroyi* is comparable or superior to that demonstrated in pigmented laboratory rats. Pattern vision may supplement or replace acoustic orientation in the detection of relatively large distant objects where the propagation losses of ultrasonic cries seriously reduce the echo intensity. Vision may be an important aid to navigation of bats on feeding or migratory flights.

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GENETIC VARIATIONS IN THE MODE OF STOLON GROWTH IN THE HYDROID, *CAMPANULARIA FLEXUOSA*[1]

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A previous investigation into the manner of stolon elongation in *Campanularia flexuosa* (Wyttenbach, 1968) elucidated a cyclic pattern of growth not previously examined in other essentially one-dimensional systems. As originally reported in stolons of *Clytia johnstoni* by Hale (1964), tips were found to elongate by means of an unending series of alternating forward surges and backward partial retractions. A detailed analysis of this pattern in a single genetic stock of *C. flexuosa* revealed that all of the major features of the growth cycle are predictable, in some respects precisely so. It also determined the effects of several environmental factors on this pattern. The present communication extends these observations to a number of additional stocks of this species for the purpose of defining the role which heritable factors may play in modifying the cycle.

In the one stock already described (Wyttenbach, 1968), it was noted that after attaining the crest (the end of a forward thrust), the stolon tip almost immediately retracted slightly (0.5–1.0 μ). Subsequently, a more extensive retraction of variable extent (up to more than 25 μ) occurred. After a brief resting interval, the tip re-extended to approximately the level of the previous crest; then, after another short resting period, it extended forward again to reach a new crest. In this particular stock, the crest-to-crest interval (cycle time) was highly consistent both from cycle to cycle in a given stolon and among different stolons, and averaged 6.15 ± 0.05 minutes. In addition, although the crest-to-crest distance (growth per cycle) routinely varied ±20% from one cycle to the next, nearly all established stolons (those at least 6 days old) grew at an average rate of 19.0–21.5 μ per cycle. Although the extent of retraction following each crest differed considerably from cycle to cycle, it was seen to change consistently from shallow ⇋ deep on a regular basis, with a period of 5 cycles. It is upon this background of information that the following observations expand.

MATERIALS AND METHODS

Observations are reported on 19 genetic stocks of *C. flexuosa*. In the present context, a genetic stock is defined as a group of colonies started with cuttings taken from the same wild colony and subcultured as necessary with cuttings from

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one of these laboratory colonies. Therefore, the several stocks observed here were distinguished as genetically different on the premise that wild colonies growing on separate pieces of substrate would be derived from different planulae even when obtained at the same general collecting site.

Colonies were established in the laboratory following the method of Crowell (1957) and are detailed in Wyttenbach (1968). The microscope slides bearing these colonies were suspended in filtered, continuously-flowing sea water adjusted to 20.0 ± 0.5° C. Feeding was carried out by placing the slides twice daily for five minutes each into a dense suspension of newly-hatched *Artemia* nauplii, a method previously found (Crowell, 1957) to provide for optimal growth. To assure that just maximally growing stolon tips were studied, only "established" stolons, those at least 6 days old, were selected for microscopic observation.

Elongation of the stolon tip was followed microscopically by measuring its position with an ocular micrometer at successive 12-second intervals throughout the observation period. By using reflected lighting and black background at a magnification of 125 ×, and by observing activities under conditions permitting the observer to maintain dark visual adaptation, such readings could be made to an estimated accuracy of ±0.3 μ.

During such observation, the colony was immersed in a dish of filtered sea water which was maintained at 20.0 ± 0.05° C by means of a set of immersed lead coils, through which flowed appropriately warmed or cooled water. In order to avoid the effects of short term variations in sea water composition on cycle time as noted earlier (Wyttenbach, 1968, p. 337), all stolons were "read" in an aliquot of the same carboy of filtered sea water set aside for the purpose at the onset of this study.

The stocks have been arbitrarily lettered in sequence, A through S, from the fastest to the slowest growing. Stock F is that previously described (Wyttenbach, 1968). Stocks E, I, M and O are male, the remainder female. In seven of them (A, F, G, M, N, O and S), at least 4 stolons each were observed microscopically, each stolon for 15 consecutive growth cycles (90–110 minutes). In five (C, D, I, J and K), 1–2 stolons each were observed, each for 8–15 cycles. Such close observation of the remaining seven stocks was limited to a single stolon each, followed for 4–5 cycles. Cycle duration was internally so consistent among all stolons timed that measurements on just 4–5 cycles give substantially as accurate a value as would have been obtained on longer continuous observations, provided that the stolon is equilibrated for at least 15 minutes at the temperature of reading before timing is initiated. All stolons were so equilibrated. In addition, the directly measured daily growth rate, as determined on several additional stolons of each of the 19 stocks, agreed closely with the values calculated on the basis of the observed cycle time and growth per cycle. Therefore, the growth per cycle and per day expressed in Figure 1 may also be considered as accurate regardless of the number of stolon growth cycles directly observed. On the other hand, reliable determination of the periodicity of rhythmic variations in the extent of retraction per cycle required continuous observation of a single stolon tip through at least 7–8 cycles, and so this period is expressed in Figure 1 only for those stocks so observed.

RESULTS

Cycle time

Figure 1A shows a range in cycle time from 6.05 to 7.18 minutes among the several stocks, with a nearly continuous distribution of times within this span. Cycle duration within each individual stock was so consistent, varying no more than ±0.2 minute from cycle to cycle in a given stolon and less than ±0.05 minute in average time among stolons, that any two colonies whose average cycle times differ by 0.10 minute or more may be concluded to be of different stock. On this basis, the 19 stocks may be subdivided into 7–8 groups. For instance, stocks G, J, P and R could not be distinguished among themselves, but they differ significantly from the 15 others.

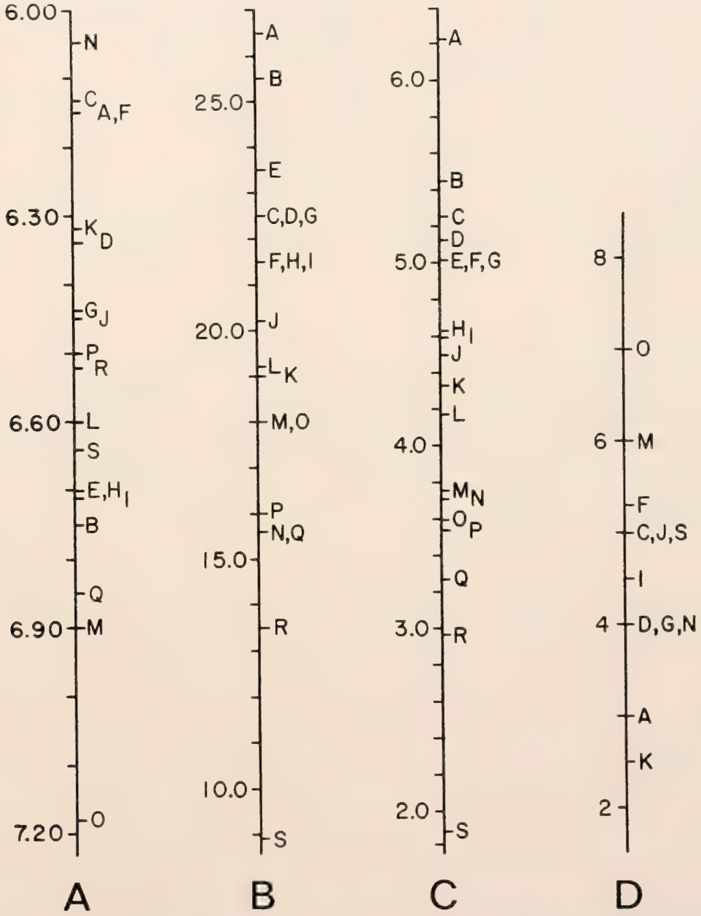

FIGURE 1. A summary of numerical data relative to stolon growth in 19 stocks (A-S) of *C. flexuosa;* (A) Average cycle duration in minutes, (B) Maximal average growth per cycle in microns, (C) Growth rate in mm per day calculated from values in (A) and (B), (D) Trough period: see explanation in text.

Although cycle duration is a factor in determining the stocks' growth rate, the rankings of the stocks on these two bases show no correlation (compare Fig. 1, A with C). Rapidly growing stock B has a longer cycle than do the two slowest growing stocks, R and S. In addition, the two stocks showing the extremes of cycle time have a virtually identical growth rate.

Growth per cycle

Much greater inter-stock variability is evident in terms of maximal average growth per cycle (Fig. 1B), which shows a 3-fold range, from 8.8 to 26.5 μ. As previously noted in stock F (Wyttenbach, 1968), and confirmed in several additional stocks here, most established stolons have an average per cycle growth at any one time of 90 to 100% of that stock's maximum. Thus by reading 3–4 such stolon tips of a given stock, the highest value obtained should be within at least 5% of the true maximum (*i.e.,* no less than 95% of maximum). In view of this, stocks differing by more than 1.0–1.5 μ per cycle may be distinguished; consequently, with respect to this variable alone, the 19 stocks may again be resolved into 7–8 groups.

The growth rate of the stolon is directly affected by its growth per cycle, and this relationship is clearly evident from a comparison of Figure 1B with 1C. A close parallel in rankings is seen here; the per cycle growth of only stocks E and X are markedly out of line with the ranking sequence of stocks by per diem growth.

Growth per unit time

The greatest variation among stocks occurs in respect to their daily growth rate (Fig. 1C), as calculated from their cycle times and growth per cycle (and corroborated by direct measurements at 24-hour intervals). A more than 3-fold range, from 1.9 to 6.2 mm, is seen between the least and most vigorous stocks. Excepting the extremes, the remaining 17 stocks display a nearly continuous spectrum from 2.95 to 5.45 mm per day.

Based upon the estimated error of no more than -5% in ascertaining the true maximal average per cycle growth of each stock and the possible error of less than $\pm 1\%$ (± 0.05 minute) in determination of its cycle time, the true maximal growth rate should be from -1% to $+6\%$ of that calculated. Thus colonies whose stolons differ by more than 0.35 to 0.40 mm per day in growth rate may be assumed to be of different stock even in the absence of direct measurements of the two component parameters. Here too then, on the basis of growth rate alone, the stocks may be segregated into 6–8 groups.

Retraction "cycle"

Although unrelated to the quantitative aspects of stolonic growth activity, the "trough period" also shows inter-stock variability. Such is illustrated in Figure 2. In Figure 2A, the stolon exhibits shallow retractions in cycles 1, 4 and 7, the others being much deeper, hence a period of 3. The stolon in Figure 2B has a period of 7, with the retractions shallowest in cycle 5, deepest in 1 (and 8). Finally, Figure 2C shows a stolon having a period of 5, with the troughs shallowest

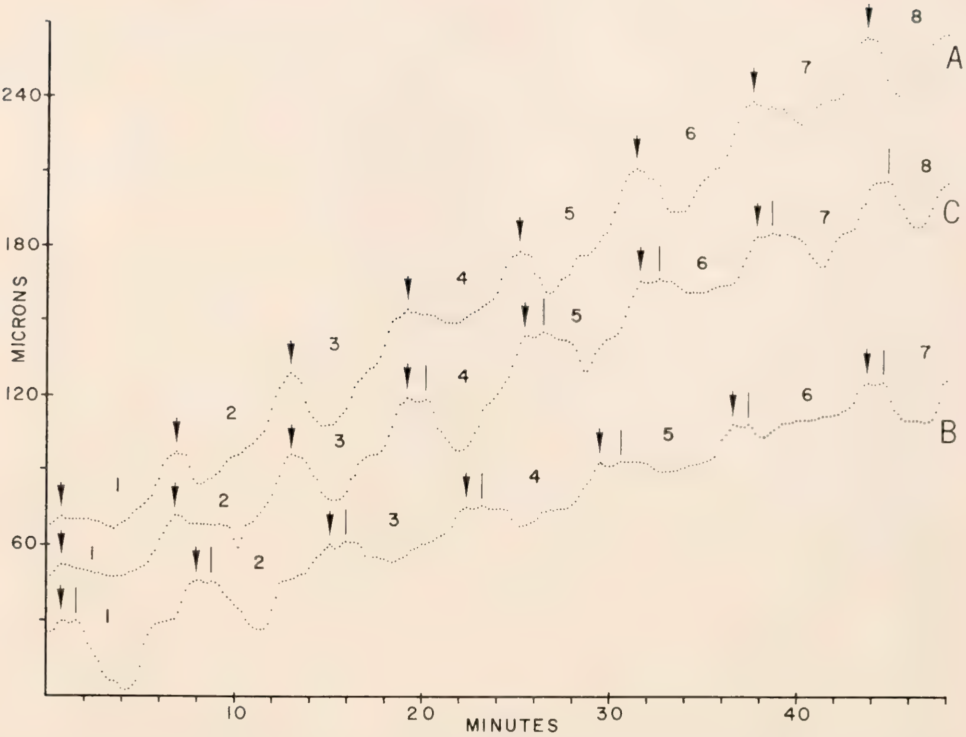

FIGURE 2. The activity of stolon tips of three different stocks. Successive dots are at 0.2 minute intervals except where a crest is reached at an odd tenth of a minute. Arrows denote the crests (primary peaks); pointer lines, the secondary peaks. Numbers 1–8 identify successive cycles, and crests (at left of number), for purposes of text description; (A) Stolon of stock A: cycle time, 6.15 minutes; average growth, 26.5 μ per cycle; trough period, 3 cycles; (B) Stolon of stock O: cycle time, 7.17 minutes; average growth, 16.3 μ per cycle; trough period, 7 cycles; (C) Stolon of stock C: cycle time, 6.14 minutes; average growth, 22.3 μ per cycle; trough period, 5 cycles.

in cycles 1 and 6, deepest in 3, 4 and 8. Figure 1D indicates the trough period of these and nine additional stocks. No consistent numerical relationship exists between this period and either cycle time or growth per cycle. Thus, this trait may serve still further to distinguish differently derived colonies.

Just as noted previously (Wyttenbach, 1968), in every stock, shortly after ingestion of a substantial meal, all retractions are largely suppressed for about 2 hours. They then gradually increase in depth to the maximum (as seen in Fig. 2) by about 8 hours after feeding.

Geometry of the growth cycle

The final feature of stolonic growth which varies among different stocks relates to the pattern of the growth cycle. As originally described (Wyttenbach, 1968) and as illustrated in Figure 2A, following each crest the tip almost immediately (usually within 0.2 minute) retracts by 0.5–1.0 μ, and this slight withdrawal is in turn followed by the more extensive retraction phase. The stolons of many stocks, however, differ by the appearance of a "secondary peak" following within 0.8 to 1.0 minute after the crest, as illustrated in Figure 2B. This secondary peak represents a resurge of the tip following its slight retraction from the crest, by which it re-extends variously almost to, to, or beyond the previous crest. Such peaks characterize many, most, or all cycles in about two-thirds of the stocks; among the others, they are only seldom seen. No attempt was made to further quantitate their frequency in particular stocks.

One variant on the secondary peak, noted in about one-fifth of the stocks, is diagrammed in Figure 2C. Here such a peak, lacking after crests 1 to 3, appears after crest 4 as a shallow resurge. In subsequent cycles it becomes increasingly dominant to the crest (or primary peak) and the latter then diminishes. By peak 8, the secondary peak has become the definitive crest as the primary peak has disappeared. Consequently, in this stolon in which all other cycles are of 6.0 or 6.2 minutes duration, the cycle of transition measured from primary peak to secondary peak is 7.2 minutes. Such "transition" cycles consitute an exception to the constancy of the cycle time as cited above. These were arbitrarily deleted in the calculation of cycle times shown in Figure 1A. However, since they do not

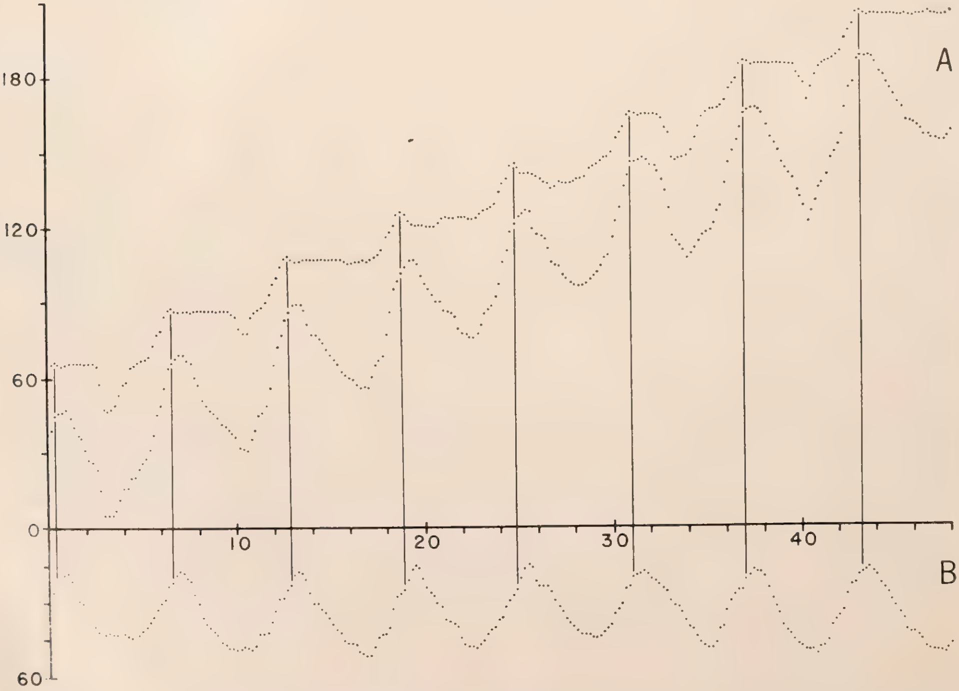

FIGURE 3. (A) Simultaneous observation of both epidermal (upper) and gastrodermal (lower) limit during elongation of a stolon tip of stock F; (B) Epidermal thickness, determined by subtracting the lower from the upper points in (A); Units: x axis, minutes; y axis, microns. Successive dots are at 0.2 minute intervals. Vertical lines align corresponding points at the time of each crest.

appear more frequently than about every 20 cycles in those stocks showing them, their effect on average cycle time would be to lengthen it by only about 0.05 minute.

Secondary peak formation is due to a modification in the relative epidermal-gastrodermal activity at the stolon tip. The epidermis has been shown (Wyttenbach, 1968) to exhibit changes in thickness which are synchronized with the growth cycle. As seen in Figure 3B, it is always thinnest, about 15 μ, at 0.4 to 0.6 minute after each crest. Then, in a stolon lacking secondary peaks, it begins to thicken almost immediately; *i.e.*, by 3–7 μ within the first 0.4 minute, or by 0.8 to 1.0 minute after the crest. Such thickening is correlated with an abrupt retraction of the same magnitude by the gastrodermis (Fig. 3A). When secondary peaks appear, either of two modifications pertains. Usually the gastrodermis retreats only slightly during the 0.4 minute after attaining its farthest advance; alternatively, in a few instances the epidermis thickens more rapidly during this time than shown in Figure 3B. In either event a secondary peak is the result of a short interval when the rate of epidermal thickening exceeds that of gastrodermal retraction; it does not denote a true advance of the coenosarc as a whole. These observations also show that although, as in Figure 3, epidermal thickening parallels gastrodermal retraction, such retraction is not a necessary condition for at least the early phase of thickening. Additional modifications which may occur when a secondary peak becomes the definitive crest have not been sought.

The uniqueness of each stock

Using the combined criteria of cycle time and growth per cycle, only two pairs of stocks are sufficiently similar to suggest identity. Stocks H and I are matched in each measurement, while C and F are within the range of normal variation from each other in each respect. Stocks H and I may not be distinguished either on the basis of trough period or the frequency of secondary peak formation, as H was not observed sufficiently to give clearcut differences from I in measurements of these values. (Yet there is no doubt that H and I differ, as they are, respectively, female and male.) Stocks C and F, however, may be separated in terms of secondary peak formation. Stock C shows secondary peaks which displace the primary peak as the definitive crest (Fig. 2C); on the other hand, stock F, in which over one thousand cycles were observed, forms such peaks only about 25% of the time, and never was one seen to supplant the primary peak as the definitive crest. On the basis of the various stolon features studied, therefore, each of the 19 stocks except H and I is distinct.

DISCUSSION

The collective observations on these several *C. flexuosa* stocks reveal that stolonic growth may differ among them in any of four variables: duration of the growth cycle, average growth per cycle, trough period, and frequency of secondary peak formation. The first two of these together determine the growth rate of the stolon tip, the latter two neither contribute directly to such growth nor show any apparent correlation with growth rate in their expression. In fact, each variable appears to be determined independent of the others.

Cycle time and average growth per cycle influence the growth per unit time disproportionately, as the relative growth rate of the stocks correlates closely with the latter but not at all with the former values. This "dominance" of per cycle growth over cycle time is due to its much greater range of inter-stock variation: the 200% higher per cycle growth of stock A over stock S is many times more than is the 20% longer cycle time of stock O over stock N. Thus stocks A and F, of similar cycle time but differing by about 5 μ in growth per cycle, show a rather large 1.2 mm difference in daily growth. Yet stocks N and Q, which are identical in growth per cycle but are 0.80 minute apart in cycle time, differ in daily growth by only about 0.40 mm.

Rhythmic variations in the depth of retraction from cycle to cycle were described and causally interpreted previously (Wyttenbach, 1968). That evidence, supported by subsequent direct visual observations in *Bougainvillia* (Wyttenbach, in preparation), indicates that the extent of retraction is determined by the nature of the hydroplasmic pressure behind the stolon tip at the time of each retraction phase. This pressure is created by sequential contractions and relaxations of a contractile zone near the stolon tip (Berrill, 1949), and it rhythmically increases and decreases due to alternatingly distal then proximal hydroplasmic flow within the stolon. Such flow follows a generally predictable time table, but *its* cycle time does not equal that for stolon growth. Therefore, the phasing between the two types of cycle is constantly changing. As the retraction phase of the growth cycle occurs at times of successively increasing, then decreasing, hydroplasmic pressure, the retractions become respectively shallower, then deeper. The trough period is therefore determined by the relative cycle times of the two interacting activities, since it represents the frequency with which a given phase relationship is repeated between them.

If the duration of the hydroplasmic flow cycle were the same in all stocks, with variations in the trough period reflecting only inter-stock differences in length of the growth cycle, then stocks with similar growth cycle times would have the same trough period. Such is not the case, as may be seen by comparing Figure 1A with 1D. Consequently, one may conclude that variations in trough period among the stocks reflect differences among them in the average duration of the hydroplasmic flow cycle.

It was previously noted (Wyttenbach, 1968) that despite maintenance of an apparently uniform environment during microscopic observation, there were intervals of days when the cycle time of the stock studied (the present stock F) lengthened by 0.1–0.2 minute. Although the responsible factors are not known, they do *not* provide a basis for the diversity of cycle times among stocks as noted here. Fortuitously, all stocks were observed entirely or in part on days when stock F showed the 6.15 minute cycle. Where some stolons of a few stocks were studied on days of lengthened cycle time in stock F, these also displayed a correspondingly longer interval. Using stock F as a control, it was thus possible to correct these times relative to a figure of 6.15 minutes for F. All values cited in Figure 1A therefore share a common base line.

The four parameters of stolonic acivity described here by no means exhaust the respects in which various *C. flexuosa* stocks may differ. For instance, *Campanularia* pedicels elongate in a fashion similar to stolons (Wyttenbach, Crowell and Suddith, 1965) and it has been found (Wyttenbach, unpublished) that here too there are inter-stock differences in both cycle time and growth per cycle. Also, the pedicel values may not be predicted from knowing the corresponding stolon values, as they are independently determined. Several additional variable features of colony growth have also been noted, though not measured critically. Thus numerous characteristics of *Campanularia* show inter-stock variation.

As the inter-stock differences observed here are consistent, and the stocks vary only in respect to their initial derivation from separate wild colonies, it is not unlikely that the differences are genetically based. Three of the variables considered, the stolon cycle time, the average growth per cycle, and the cycle time of hydroplasmic flow, are sufficiently easily measured and quantified that they may be amenable to an analysis of their mode of inheritance. The only previous studies of genetic transmission of traits in hydroids are those by Hauenschild (1954, 1956), who concerned himself with the capacity for stolon fusion as expressed in *Hydractinia*. Such fusion occurs between stolons of some pairs of colonies (stocks) but not others, and he has investigated the inheritance of these tissue "compatibility" factors.

Technically, genetic studies in *C. flexuosa* are feasible. In laboratory culture, colonies routinely form numerous gonangia which produce mature gametes continuously for several days or weeks. In addition, young colonies derived from metamorphosed planulae have often been seen when male and female stocks were maintained side-by-side.

Two potential difficulties exist. For one, the stolon cycle duration and growth per cycle, and possibly also the hydroplasmic flow cycle time, vary among the stocks almost continuously between the extremes noted, suggesting that these features may be polygenic in determination and thus more difficult to analyze genetically. Also, since these stocks are not inbred, *i.e.*, are not genetically homozygous, interpretation of the results must be viewed with the realization that diverse genetic backgrounds might affect the expression of genes determining the trait considered. Still, suitably designed crosses should provide insight into the inheritance of traits both related and unrelated to stolonic growth.

I should like to acknowledge the very fine technical assistance provided by Sandra Collins Thompson. My appreciation is extended also to Dr. Sears Crowell for his critical reading of the manuscript.

SUMMARY

Comparative observations of stolon elongation in 19 genetic stocks of *C. flexuosa* reveal four respects in which such activity may differ, with each varying independently of the others. Two of these determine the stolonic growth rate: the duration of the growth cycle and the average growth per cycle. The stocks show a nearly continuous spectrum of these values, ranging, respectively, from 6.05 to 7.18 minutes and from maxima of 8.8 to 26.5 μ. The resulting maximal daily growth of the stocks varies from 1.9 to 6.2 mm. This rate is influenced much more by the stolon's growth per cycle than by its cycle time.

The stocks vary also in the frequency of their repeating pattern of retractions from cycle to cycle; this "trough period" ranges from 2.5 to 7. Thus indirectly

it may be concluded that the duration of each cycle of back and forth hydroplasmic flow in the stolon is also an inter-stock variable.

Finally, the stolons of many stocks show a secondary forward surge of the tip following shortly after the crest of the cycle is reached, and the frequency of such "secondary peak" formation is also a stock characteristic. Its anatomical basis is cited.

With respect to these four parameters of stolon growth activity alone, 18 of the 19 stocks are distinct from one another. It is suggested that several of these traits are so constant and easily measured that studies into their mode of inheritance should be feasible.

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Communications relative to manuscripts should be sent to Dr. W. D. Russell-Hunter, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between May 23 and September 1, and to Dr. W. D. Russell-Hunter, P.O. Box 103, University Station, Syracuse, New York 13210, during the remainder of the year.

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INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers, preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. ***Manuscripts.*** Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes in the body of the text should also be avoided unless they are absolutely necessary, and the material incorporated into the text. Text foot-notes should be *numbered* consecutively and typed *double*-spaced on a separate sheet. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. ***A condensed title*** or running head of no more than 35 letters and spaces should be included.

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